nature materials

SUPPLEMENTARY INFORMATION

DOI: 10.1038/NMAT3787

Water-mediated structuring of bone apatite

Yan Wang[1†], Stanislas Von Euw[1†], Francisco M. Fernandes[1], Sophie Cassaignon[1], Mohamed Selmane[1], Guillaume Laurent[1], Gérard Pehau-Arnaudet[2], Cristina Coelho[3], Laure Bonhomme-Coury[1], Marie-Madeleine Giraud-Guille[1], Florence Babonneau[1], Thierry Azaïs[1]* and Nadine Nassif[1]*

† These authors contributed equally to this work

DOI: 10.1038/NMAT3787

Methods

Sample preparation

Bone sampling:

Fresh bone. Samples were harvested from two-year-old healthy French ewes. Bone was extracted from the proximal part of the diaphysis and distal epiphysis of humerus and femur. The work plan was reviewed and approved by the IMM Recherche's Institutional Animal Care and Use Committee (IACUC) prior to the initiation of this study. The animal research center (IMM-Recherche) received an agreement (n°75-14-01) on September 08th, 2008 for a period of 5 years by the "Sous-Direction de la Protection Sanitaire" of the French Authorities.

Dry bone. The extracted untreated bone was dried for 24 hours under sterile conditions, *i.e.* under laminar flow hood, at ambient temperature.

D_2O exchanged sheep bone. Fresh bone sample was soaked in D_2O (99.9% Sigma Aldrich) for 5 days at 37°C. D_2O was renewed every day.

Extracted bone mineral. The organic matrix (collagen and other proteins) was removed from the above fresh bone sample by immersion in dilute NaClO aqueous solution treatment based on the procedure described by Weiner & Price[1]. A bone fragment (~150 mg) was shredded with a scalpel prior to being grinded in an agate mortar. Then, the ground bone underwent the following treatments: sonication in a cold dilute NaClO aqueous solution (10 ml, pH ~9.6), centrifugation and grinding. The NaClO was renewed after each grinding step. This procedure (referred as method 2 in the paper) was performed three times. Overall, the sonication treatment with sodium hypochlorite solution lasted 4 hours. The sample was washed three times in distilled water (10 ml) and twice with 95% ethanol (10 ml). Before drying for three days at 37 °C, the sample was sonicated for 15 min in 100% ethanol (2 ml).

Synthetic apatite. $CaCl_2.2H_2O$ (calcium chloride dihydrate, ≥ 99 %), $NaH_2PO_4.H_2O$ (sodium phosphate monohydrate, ≥ 99.1%), $NaHCO_3$ (sodium bicarbonate, ≥ 99.5%), $Ca(NO_3)_2.4H_2O$ (calcium nitrate tetrahydrate, ≥ 99.0 %) and $(NH_4)_2HPO_4$ (diammonium hydrogenphosphate, ≥ 99.99%) were purchased from Sigma-Aldrich.

CHA was prepared following the synthesis described by Nassif *et al*.[2] (referred as HA-2 in the referenced paper). Experiments were carried out at room temperature (22 ± 1°C). A solution containing 110 mM $CaCl_2$, 33 mM NaH_2PO_4, and 33 mM $NaHCO_3$ was prepared in 500 mM acetic acid consistent with the synthesis of preferentially B-type CHA, which has a formula of $Ca_{10-x}(PO4)_{6-x}(CO_3)_x(OH)_{2-x}$ with $0 \leq x \leq 2$. The pH was adjusted to 2.2 with hydrochloric acid. Two flasks (35 mL, h = 50 mm) containing these solutions (20 mL) and covered by perforated Parafilm (to slow down the gas diffusion) were placed into a closed chamber (1000 cm^3). The CHA precipitation was triggered *via* the slow increase of the pH of the solution caused by the vapors of fresh ammonia aqueous solution (30% w/w, 8 mL) in the chamber. A few hours after ammonia introduction, precipitation occurs in the solution. After 6 days the gas diffusion was considered complete (pH~10-11), and the solids were washed and centrifuged (6000 rpm, 10 min) firstly in distilled water and then in ethanol to remove the non-precipitated salts. The recovered crystals were dried at 37°C for 7 days before characterization.

^{13}C-CHA:* the procedure used was identical to that performed with CHA except for $NaH^{13}CO_3$ (sodium bicarbonate, enriched with 98% of ^{13}C) which was purchased from Cortecnet.

HCA was prepared following the synthesis described by Takemoto *et al*.[3] Briefly, 0.3 M of an $(NH_4)_2HPO_4$ aqueous solution (100 mL) for which the pH was fixed at 10 by addition of NH_4OH (28 %wt in aqueous solution) was added to 0.5 M of $Ca(NO_3)_2.4H_2O$ solution (100 mL) at room temperature under stirring at a feeding rate of 3 mL/min. After 24 h stirring, the

DOI: 10.1038/NMAT3787

calcium phosphates were separated from the suspension by centrifugation, washed with distilled water then dried at 105°C for 24h.

^{43}Ca-CHA*: was prepared following the synthesis described by Nassif *et al.*[2] (referred as HA-1 in the paper). However, $CaCO_3$ being the only commercial source of ^{43}Ca available, some adjustments were carried out. ^{43}Ca*-CHA was prepared from the precursor $CaCO_3$ for the calcium (instead of $CaCl_2.2H_2O$); $NaH_2PO_4.H_2O$ remaining the precursor of orthophosphates. Briefly, 66 mg of $CaCO_3$ (enriched with 65% of ^{43}Ca from Cortecnet) was dissolved in 20 mL aqueous solution. In order to remove carbonates, the pH of this solution was lowered to pH 3 (with hydrochloric acid solution 27 %wt added dropwise), followed by degassing with argon for a period of 90 min; avoiding the precipitation of any allotropic form of $CaCO_3$. Subsequently, 54.5 mg of $NaH_2PO_4.H_2O$ were also dissolved in the previous solution, attaining a calcium-to-phosphate (Ca/P) molar ratio of 1.67. The pH was then adjusted to 2.2, and the remaining procedure kept as in the referred article.

CHA-SBF was precipitated from a 1.5 simulated body fluid solution (1.5 SBF), *i.e.* characterized by 1.5 times higher ion concentration than human blood plasma. This 1.5 SBF solution was prepared according to the procedure described by Rhee *et al.*[4], *i.e.* by dissolving at 37°C the following reagents: NaCl (11.99 g), $NaHCO_3$ (0.529 g), KCl (0.335 g), $K_2HPO_4.3H_2O$ (0.342 g), $MgCl_2.6H_2O$ (0.457 g), $CaCl_2$ (0.416 g), Na_2SO_4 (0.107 g) in 1 L double distilled water buffered at pH 7.4 with 1.2 g tris(hydroxymethyl) aminomethane and few drops of hydrochloric acid. The SBF solution was brought to -20°C. This fast quenching leaded to the formation of calcium phosphates precipitation. After 24 h at -20°C, the frozen solution thawed at room temperature. Then solids were washed and centrifuged (6000 rpm, 10 min) 3 times in distilled water and then dried at 37°C for 7 days before characterization.

CHA-200: CHA powder was spread in a Petri dish and then placed in an oven at 200°C for 5 days.

CHA-NaOH and CHA-SBF-NaOH: CHA or CHA-SBF was dispersed in NaOH aqueous solution (0.2 wt.%) at pH 14. The mixture was kept under continuous moderate stirring for a period of 12 and 7 days respectively.

Synthetic ACP. ACP was synthesized according to the Heughebaert method[5]. Technically, 46.3 g of $Ca(NO_3)_2.4H_2O$ and 27.2 g of $(NH_4)_2HPO_4$ were dissolved in 0.55 L aqueous solution of ammonia of approximately 1.1 M and 1.3 L aqueous solution of ammonia of approximately 0.5 M respectively. The calcium solution was then poured into the orthophosphate solution forming a precipitate which was immediately filtered through a Büchner funnel. The recovered precipitate was firstly washed with an aqueous solution of ammonia of approximately 0.08 M, frozen in liquid N_2 and lyophilized for a period of 72 h, and lastly, stored at -20°C.

H_2O wet powders (CHA, CHA-SBF, CHA-200, CHA-NaOH, CHA-SBF-NaOH, HCA, extracted bone mineral, ACP). The 4 mm rotor was filled with powder (50 mg aprox.) then soaked with 10 µL of double distilled H_2O.

Sample Characterization

^{1}H, ^{31}P and ^{13}C solid state NMR experiments were recorded on a Avance 300 Bruker spectrometer operating at (^{1}H) = 300.13 MHz, (^{31}P) = 121.5 MHz and (^{13}C) = 75.5 MHz. For fresh bone, small pieces (3x3x3 mm^3 aprox.) where placed in-between two Teflon spacers into a 4 mm (O.D.) NMR rotor. The NMR study was performed within 2 hours after extraction from untreated bone. All the other samples were placed in-between Teflon spacers

DOI: 10.1038/NMAT3787

into a 4 mm (O.D.) zirconia rotor and spun at 8 kHz. $t_{90°}(^{1}H)$ and $t_{90°}(^{31}P)$ were 4.5 µs and 5.5 µs, respectively. ^{1}H and ^{13}C chemical shifts were referenced (δ = 0 ppm) to TMS and ^{31}P chemical shift was referenced to 85 wt.% aqueous H_3PO_4. ^{1}H-^{31}P HetCor experiments parameters were the followings: (*i*) bone: recycle delay RD = 3.5 s, contact time CT = 10 ms, 400 transients for each 128 t_1 increments; (*ii*) CHA and HCA: RD = 2 s, contact time CT = 1 ms, 232 transients for each 128 t_1 increments; (*iii*) ACP: RD = 3 s, contact time CT = 1 ms, 128 t_1 increments and 232 or 8 transients, for dry and wet ACP respectively. For wet ACP, the experimental time is considerably shorter in order to avoid the transformation of ACP into apatite and OCP during the NMR experiment. Variable contact time ^{1}H-^{31}P HetCor experiments on bone were recorded with CT = 100 µs, 250 µs, 500 µs, 1 ms, 2 ms, 4 ms and 10 ms. ^{1}H-^{13}C HetCor experiments parameters were RD = 3.5 s, contact time CT = 1 ms, 800 transients for each 80 t_1 increments. ^{43}Ca (I = 7/2) is the active isotope of calcium but its natural abundance (0.135 %) prevents the convenient use of ^{43}Ca NMR in natural abundance conditions. For these reasons, we studied a 60% ^{43}Ca-enriched CHA sample (^{43}Ca*-CHA). ^{1}H-{^{43}Ca} TRAPDOR experiments were recorded on a Avance 700 Bruker spectrometer operating at (^{1}H) = 700.0 MHz, (^{43}Ca) = 47.1 MHz. The sample was placed into a 3.2 mm (O.D.) zirconia rotor and spun at 22 kHz. The following parameters were used: RD = 5 s, 1080 transients and a RF field (^{43}Ca) = 25 kHz applied during 10 rotor periods. The ^{31}P-filtered ^{1}H spectra were recorded through a ^{1}H-^{31}P-^{1}H Double CP MAS experiment[6] with a first CT_1 = 1 ms and a second CT_2 = 850 µs in order to maximize the 5-15 ppm resonances of the ^{1}H spectra compared to the hydroxyls resonance. The ^{31}P-filtered ^{1}H EXSY experiments were recorded through a first block of ^{1}H-^{31}P-^{1}H Double CP MAS experiment (CT_1 = 1 ms; CT_2 = 850 µs) in order to maximize the 5-15 ppm resonances of the ^{1}H spectra compared to the hydroxyls resonance followed by a standard EXSY experiment (mixing time = 10 μs and

10 ms). To ensure an efficient ^{1}H spin diffusion process, the experiments were conducted on dry bone.

CryoTEM observations were performed on a Jeol 2010F operating at 200 kV under low dose conditions (15 electrons/Å2/second) using a Gatan (USA) ultrascan 4000 camera. Powder samples were suspended in double distilled water, sonicated for 10 min then placed under gentle stirring for 1 hour. One drop was then deposited on a R2/2 Quantifoil grid (Germany). The grid was cryofixed in liquid ethane using a cryo-fixation device (EMGP, Leica, Austria). Samples were transferred inside the microscope with a Gatan 626DH cryoholder (USA).

For TEM studies, the nanoparticles (CHA, CHA-SBF or HCA) were dispersed either in ethanol (referred as "standard TEM" in the text) or water (*i.e.* the preparation was similar to that described in the CryoTEM section). Few drops of the resulting dispersion were deposited on a carbon coated copper grid. After solvent evaporation, TEM investigations were performed with a FEI TECMAI G2 Spirit Twin electron microscope operating at 120 kV. For the native sheep bone, fresh bone samples were fixed in 2.5% glutaraldehyde. After washing in a cacodylate/saccharose buffer solution (0.05 M/0.6 M, pH 7.4), the bone samples were post-fixed with 2% osmium tetroxyde in the buffer solution for 1 h at 4°C, washed, dehydrated through successive ethanol baths (50%, 70%, 95%, and 100%) and embedded in araldite for ultrathin sectioning (Ultracut Reichert Young). TEM investigations were performed on thin sections (~80 nm) deposited on copper grids but without staining. HRTEM investigations were performed with a JEOL JEM-2010 equipped with a LaB_6 gun operating at 200kV.

For the Wide Angle X-ray diffraction experiments (WAXS), the dry powders were directly inserted into X-ray cylindrical borosilicate capillary tubes (1 mm in diameter). Two procedures were used for each wet sample. Dry powders were soaked with few mL of double distilled water before (similarly as described in the CryoTEM section) or after being inserted

DOI: 10.1038/NMAT3787

into X-ray capillary tubes to check the occurrence of possible orientation of the particles through a capillary confinement effect generated by the hydration step. When compared, the resulting diffractograms appear similar. Afterwards, the tubes were flame-sealed to keep the samples hydrated and then placed directly in the vacuum chamber beam. X-ray diffraction experiments were performed with a S-MAX 3000 RIGAKU device using a monochromatic CuKα1 radiation. The data were collected in the 10-60° range (2θ). The sample-to-detector distance was 58.6 mm. Two-dimensional WAXS patterns were collected with image plates (15x15 cm^2) then scanned with a 50 µm resolution. The diameter of the cylindrical beam dimension at the specimen was 400 μm. The raw data were analyzed with Fit2D (A. Hammersley) and FullProf software.

Powder X-ray diffraction (XRD) diagrams were carried out using a Bruker D8 X-ray diffractometer operating in the reflection mode at CuKα radiation with 40 kV beam voltage and 40 mA beam current. The data were collected in the 5-60° range (2θ) with steps of 0.02° and a counting time of 2 s.

Zeta potential measurements were carried out using a Malvern Zetasizer Nano ZS90. Each apatite powder was dispersed in water (approx. 0.02 %wt) by sonication for 10 minutes and analyzed three times between 12 and 34 runs until reaching convergence (automatically set by the instrument software between preset limits of 10 and 100 runs). Phase plots were analyzed to ensure robustness of each analysis. Each sample was analysed at 20, 60 and 120 minutes after sonication. Acidity of the suspensions was determined simultaneously with zeta potential measurements.

Dynamic Water Sorption of apatite samples was carried out in an Aquadyne DVS-2 gravimetric water sorption analyser (Quanthachrome Instruments). Samples were dehydrated overnight at 120 °C under mild vacuum (10^{-2} bar approximately) followed by insertion on the

analyser pans and gravimetric stabilization at 80°C and 0% relative humidity. Isotherms were conducted at 60 °C and 37 °C between 0 and 95% RH.

Thermogravimetric analysis (TGA) was performed on a thermo-microbalance instrument (NETZSCH STA 409PC) with the synthetic mineralized matrices. The measurement was performed from room temperature to 1000°C in an oxidizing atmosphere with a heating rate of 5°C/min.

DOI: 10.1038/NMAT3787

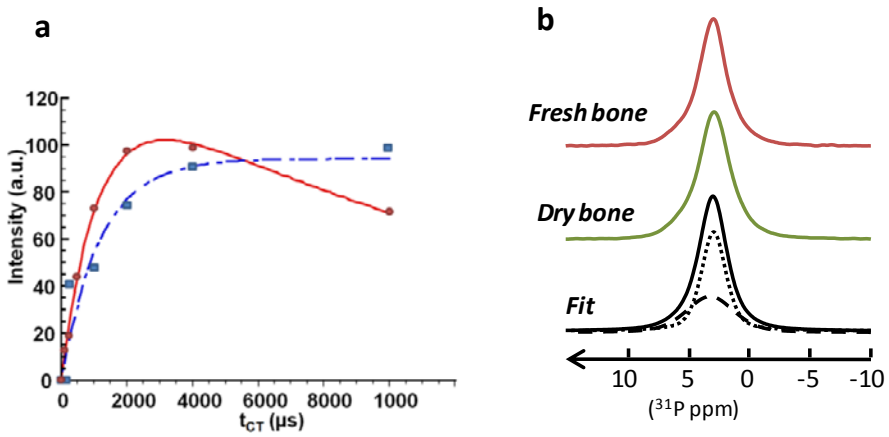

Figure S1/ (**a**) Variable contact time ^{1}H-^{31}P CP experiment displaying the evolution of the ^{31}P signal intensity of phosphate correlating with the hydroxyls (blue curve) and with the water resonance (red curve) obtained through 2D ^{1}H-^{31}P HetCor experiments of fresh bone (CT = 100 µs, 250 µs, 500 µs, 1 ms, 2 ms, 4 ms and 10 ms). The fitting of the curves was done according to the I-S model[7] using the following equation: $I(t_{CT}) = I_0.(1-T_{CP}/T_{1\rho})-1[\exp(-t_{CT}/T_{1\rho})-\exp(-t_{CT}/T_{CP})]$ where I_0 is the Zeeman intensity, T_{CP} is the cross relaxation time constant and $T_{1\rho}$ is the relaxation time of protons in the rotating frame. A stronger dipolar coupling was revealed between adsorbed water and phosphate from the surface layer (T_{CP} = 1.10 ms) compared to the coupling between hydroxyls and phosphates from the bulk (T_{CP} = 1.18 ms). (**b**) The ^{31}P MAS spectra recorded on hydrated (red) and dry (green) samples of bone are identical, showing that the overall phosphate content and environment in bone mineral is not modified upon dehydration and in particular that there is no alteration of the surface layer during this process. The fitting of the resulting spectra (black curve) by the line shapes of the two components detected by 2D HetCor experiments yields the proportion of the apatitic core (dotted line) *vs*. the surface layer (dashed line) for the bone sample: 55 vs. 45±5 %, respectively.

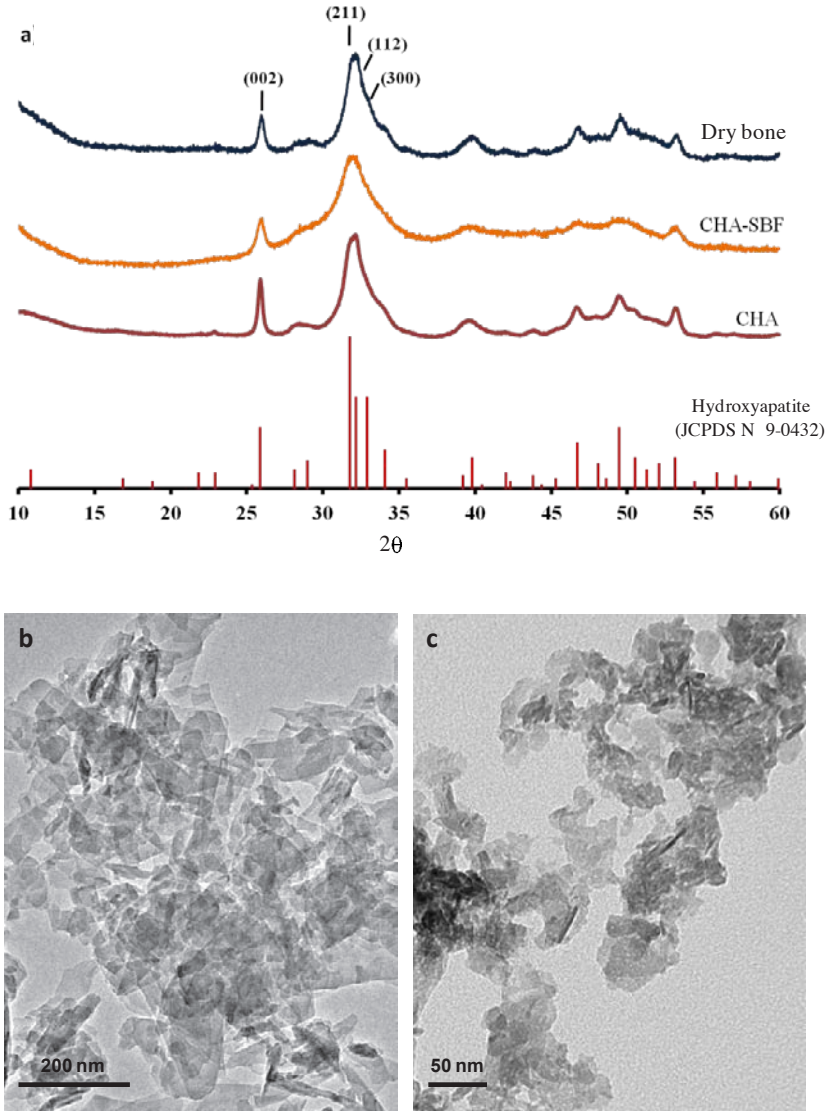

Figure S2/ The samples CHA and CHA-SBF are biomimetic apatites. The CHA and CHA-SBF are apatite samples possessing a low degree of crystalinity similar to the bone mineral as shown by XRD (no contaminant phase is detected) (**a**). Standard TEM observations of CHA and CHA-SBF powders. They are formed of nanometric platelets as bone mineral. The size is polydispersed for CHA with an average of 200×100×5 nm^3 (**b**) whereas CHA-SBF platelets are smaller and more homogeneous in size with an average of 50×50×2-4 nm^3 (**c**).

DOI: 10.1038/NMAT3787

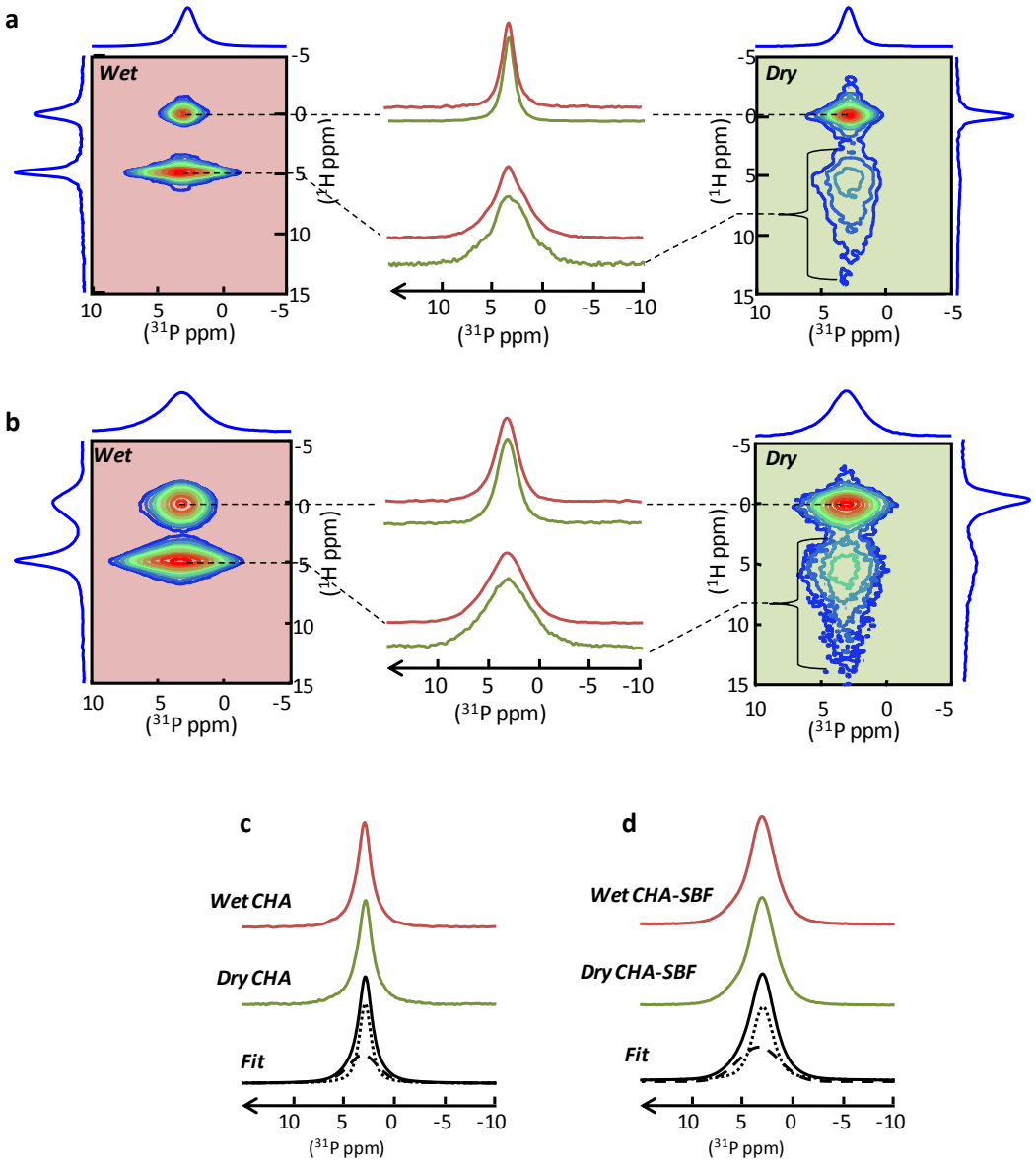

Figure S3/ a-b, 2D ^{1}H-^{31}P HetCor experiments (contact time CT = 1 ms) of CHA (**a**) and CHA-SBF (**b**) particles in dry (green) and H_2O soaked (red) conditions. Two correlation peaks are detected and correspond to (*i*) ^{31}P from the apatitic core (signing with the hydroxyls at $\delta(^{1}H)$ = 0 ppm) and (*ii*) ^{31}P at the hydrophilic surface which is signing with the sharp resonance of adsorbed water molecules ($\delta(^{1}H)$ = 4.85 ppm) in the hydrated state and with the broader resonance of POH and residual water molecules (5-15 ppm) in the dry state. **c-d**, The ^{31}P MAS spectra recorded on hydrated (red) and dry (green) CHA (**c**) and CHA-SBF (**d**) are

identical, showing that the overall phosphate content and environment in biomimetic apatites is not modified upon dehydration and, in particular, that there is no alteration of the surface layer during this process. The fitting of the resulting spectra (black curve) by the line shapes of the two components detected by 2D HetCor experiments yields the proportion of the apatitic core (dotted line) *vs*. the surface layer (dashed line) for CHA (60 vs. 40±5 %, respectively) and CHA-SBF (55 vs. 45±5 %, respectively).

DOI: 10.1038/NMAT3787

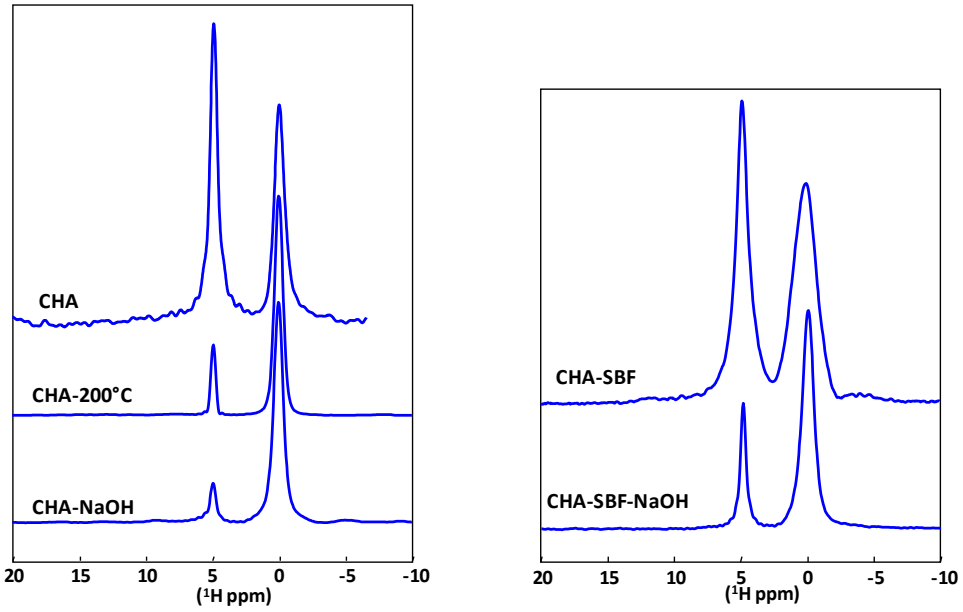

Figure S4/ ^{31}P-filtered ^{1}H NMR spectra of wetted powders show a subsequent reduction of the ACP layer after either a thermal or a chemical treatment of the biomimetic particles (CHA (left) and CHA-SBF (right)). The removal of the disordered layer is ~90% and ~80% for CHA treated with alkaline solution and treated at 200°C, respectively, and ~65% for CHA-SBF treated with alkaline solution. This value is obtained by fitting the water resonance at 4.85 ppm after normalization of the hydroxyl resonance at 0 ppm.

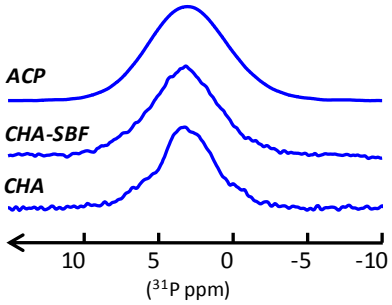

Figure S5/ The ^{31}P MAS spectrum of ACP is similar in terms of position and linewidth to the ^{31}P resonance of the outer layer of CHA and CHA-SBF particles highlighting that the surface mineral layer of biomimetic apatite is related to an amorphous calcium phosphate phase as found for bone mineral.

DOI: 10.1038/NMAT3787

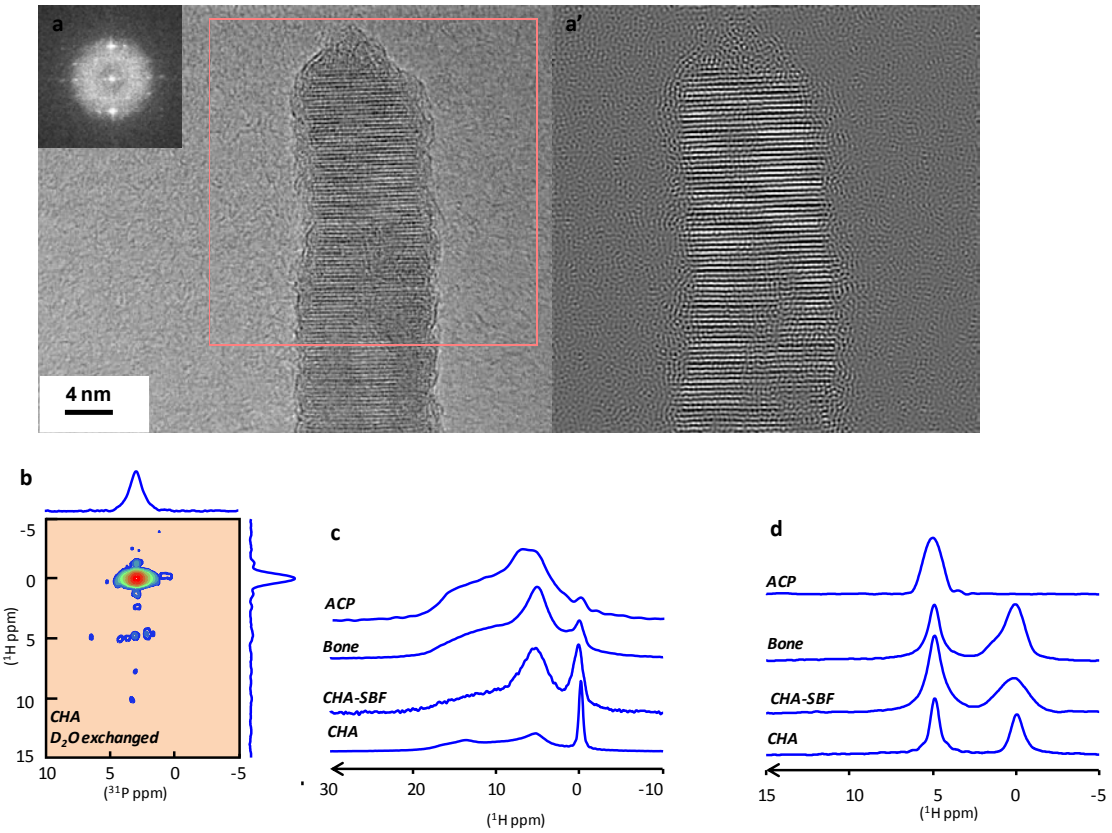

Figure S6/ (**a**) HRTEM micrograph of CHA particle exhibiting the (002) planes as determined from the 2D Fast-Fourrier Tranform (FFT) depicted in the inset, along with an outer non crystalline layer attributed to the amorphous hydrophilic layer. The inverse FFT (**a'**) performed on the area marked in HRTEM reveals crystalline domains along with disordered domains. A possible denaturation under the HRTEM electron beam cannot be discarded. (**b**) The ^{1}H-^{31}P HetCor spectrum of D_2O exchanged CHA displays solely the resonance of phosphates correlating with ^{1}H from the hydroxyls. Thus protons from adsorbed water molecules are involved in a chemical exchange with the protons of the surface mineral layer as the broad resonance of residual H_2O and HPO_4^{2-} (5-15 ppm) is absent from the 2D spectrum. **c**-**d**, The comparison between the ^{31}P-filtred ^{1}H spectra of dry and wet samples is particularly informative and summarizes the similar behaviour upon hydration/dehydration of ACP, CHA-SBF and the bone samples. In the dry state, they exhibit a broad component spanning the same range of chemical shift ($\delta(^{1}H)$ = 5-15 ppm) (**c**). For fully hydrated samples

(fresh bone, wet CHA-SBF and wet ACP), the broad components merge into a single sharp resonance at $\delta(^1H)$ = 4.85 ppm (**d**). The broad component is no longer present in the spectra of wet samples as it is neither hidden in the baseline nor masked by the adsorbed water resonance which is characteristic of a chemical exchange phenomenon which involves HPO_4^{2-} ions and residual H_2O molecules.

DOI: 10.1038/NMAT3787

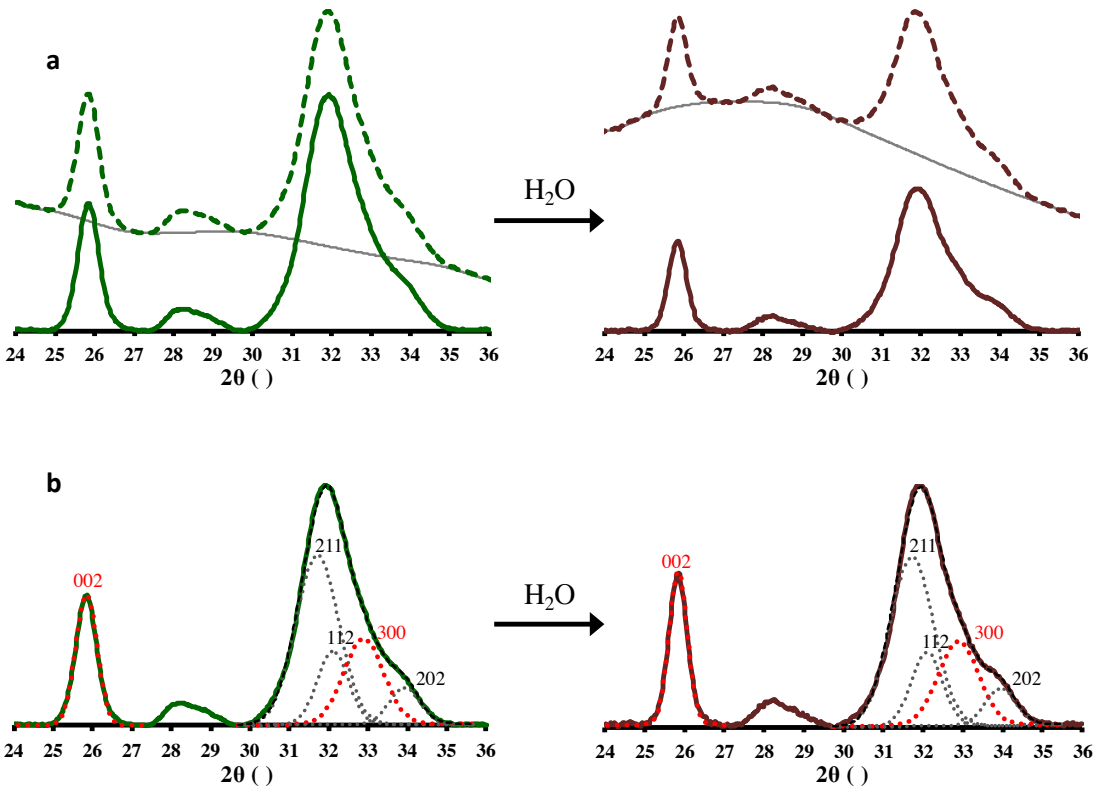

Figure S7/ (**a**) 1D radial average of the WAXS patterns of dry (left) and wet (right) CHA powder with (red) and without (blue) baseline correction. (**b**) Fitting of the 1D radial average of the WAXS patterns corresponding to dry (left) and wet (right) CHA powder using five line shapes corresponding to the (002), (211), (112), (300) and (202) reflections. The fitting was done by varying the intensities but by keeping constant the positions and linewidths. The resulting area ratios (002)/(300) are 1.01 and 1.37 for dry and wet CHA powder, respectively. Thus, the intensity increase of the (002) reflection relative to the orthogonal (300) reflection (35 %) indicates the orientation of the platelets in the *c*- axis direction.

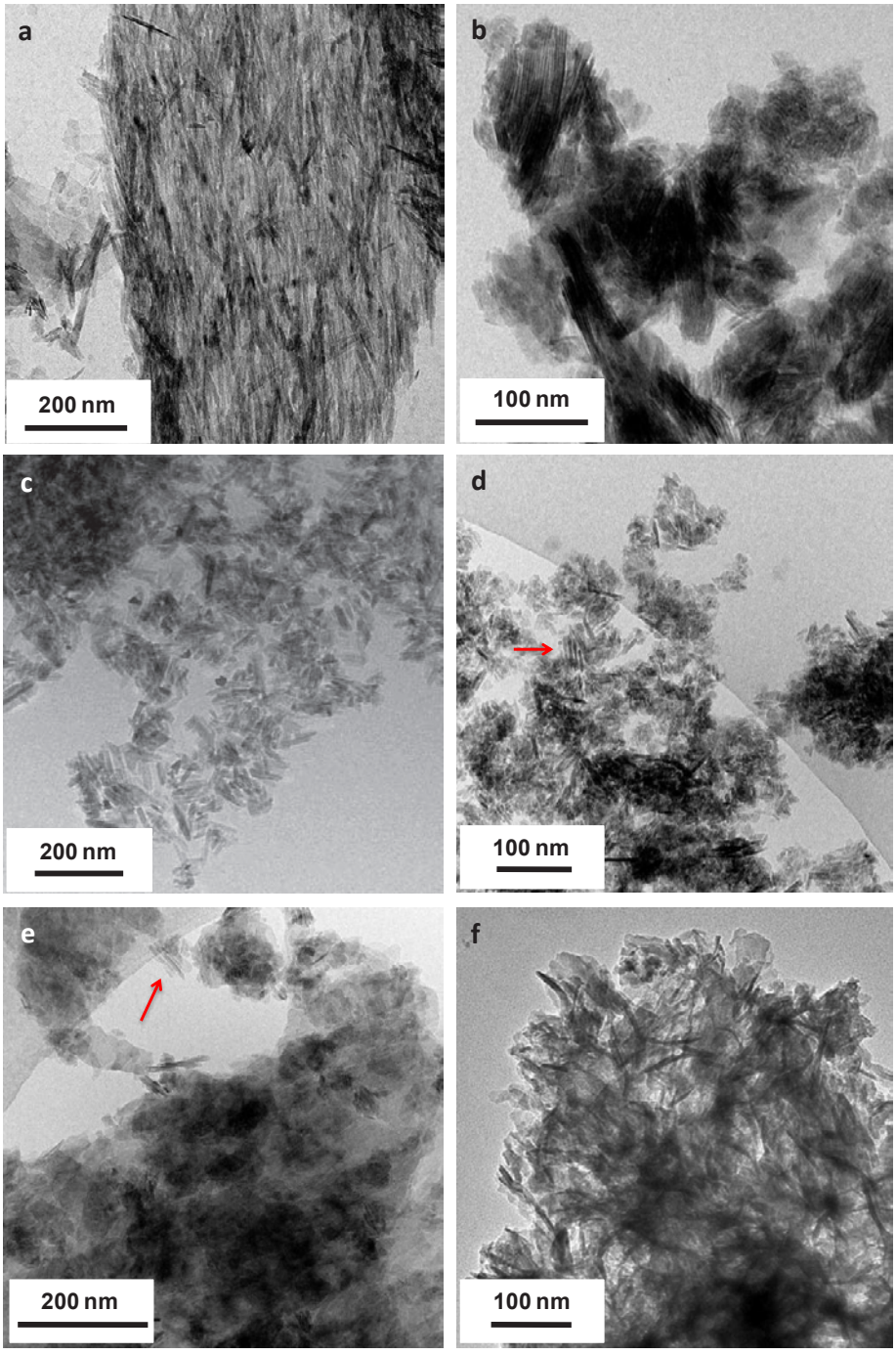

Figure S8/ a-e, CryoTEM pictures of CHA (**a**), CHA-SBF (**b**), HCA (**c**), CHA-SBF-NaOH (**d**), CHA-NaOH (**e**). **f**, Standard TEM picture of CHA-SBF particles dispersed in water. Red arrows in d and e indicate few aggregates of apatite particles.

DOI: 10.1038/NMAT3787

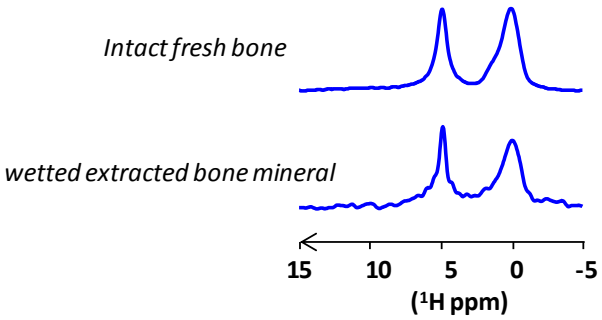

Figure S9/ ^{31}P-filtered ^{1}H NMR spectra of intact fresh bone and wetted extracted bone mineral showing the preservation of the hydrated ACP-like layer.

References

1. Weiner, S. & Price, P.A. Disaggregation of bone into crystals. *Calcif. Tissue Int.* **39**, 365-375 (1986).
2. Nassif, N. *et al.* In vivo inspired conditions to synthesize biomimetic hydroxyapatite. *Chem. Mater.* **22**, 3653-3663 (2010).
3. Takemoto, S. *et al.* Selective protein adsorption and blood compatibility of hydroxy-carbonate apatites. *J. Biomed. Mater. Res. Part A* **69A**, 544-551 (2004).
4. Rhee, S.H. & Tanaka, J. Hydroxyapatite formation on cellulose cloth induced by citric acid. *J. Mater. Sci.: Mater. Med.* **11**, 449-452 (2000).
5. Somrani, S., Banu, M., Jemal, M. & Rey, C. Physico-chemical and thermochemical studies of the hydrolytic conversion of amorphous tricalcium phosphate into apatite. *J. Solid State Chem.* **178**, 1337-1348 (2005).
6. Folliet, N. *et al.* Investigation of the interface in silica-encapsulated liposomes by combining solid state NMR and first principles calculations. *J. Am. Chem. Soc.* **133**, 16815-16827 (2011).
7. Pines, A., Gibby, M. & Waugh, J. Proton-enhanced NMR of dilute spins in solids. *J. Chem. Phys.* **59**, 569-590 (1973).