Supplementary Information

Impact of Collagen Confinement *vs.* Ionic Substitutions on the local disorder in Bone Apatites

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Materials and Methods

Synthesis of apatites and hybrid models:

CHA: The nanosized carbonated HAP (CO_3^{2-} content ~ 5 wt.% from TGA) was obtained at ambient temperature by an ammonia vapors diffusion method[1]. Briefly, an aqueous solution of $CaCl_2$, NaH_2PO_4, and $NaHCO_3$ was prepared in acetic acid (carbonate-to-phosphate molar ratio C/P = 1; Table S1). 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 solution by the vapors of a fresh ammonia aqueous solution (30 wt.%, 8 mL) also placed in the chamber. A few hours after ammonia introduction, precipitation occurs in the solution. After complete gas diffusion (pH~10), the solids were washed and centrifuged (6000 rpm, 10 min) first in distilled water and then in ethanol to remove the non-precipitated salts. The recovered crystals were dried at 37°C for 3 days before characterization.

CHA-SBF: nanosized carbonated Hap are precipitated directly from a SBF solution[2] possessing a composition close to human blood plasma. A solution 1.5 times more concentrated compared to standard SBF is prepared (1.5×SBF).[3] The composition of the solution is detailed in Table S1 (C/P = 4.2). 1 L of the solution is frozen at -20°C for 1 night. Then, the solution is thawed at 5°C. The resulting particles are recovered by centrifugation (6000 rpm, 10 min) and then dried at 37°C for 3 days before characterization. The fraction of carbonate ions is around 7 wt.%, from TGA.

Coll/CHA and Coll/CHA(SBF): For the hybrid matrices, the Hap precipitation occurred inside a dense 3D collagen matrix adopting a cholesteric organization.[4,5] *Coll/CHA*: 3 mg/mL soluble acidic collagen solution (0.5 mM acetic acid) mixed with the CHA precursor solution (0.5 mM acetic acid) was continually injected in a closed dialysis chamber in the rate range of 1µL/min-15 mL/min for 8 days. The reverse dialysis process was set against polyethylene glycol (PEG, 35 kDa, Fluka) dissolved in 0.5 M acetic acid up to ~300 mg/mL. After the total amount of solution was injected, the dialysis was continued for 4-8 days in order to obtain a homogeneous concentration in the samples. The pH was then increased to 9-10 by ammonia gas diffusion for 4-8 days to induce collagen fibrillogenesis together with the Hap precipitation. The degree of mineralization of the resulting hybrid matrix is about 5 wt.% from TGA. The mineral was mainly deposited into gap regions of the collagen fibrils. *Coll/CHA(SBF)*: The mineral charge of the previous matrix was increased at 37°C under mild rotative stirring (200 rpm) by immersion in the 1.5×SBF solution (45 mL). The 1.5×SBF solution was renewed every 4 days for 16 days. The

degree of mineralization of the resulting hybrid matrix is about 50 w% close to the value determined for compact bone.

cHA-X: The synthesis has been realized following the procedures described in the literature.[6] The water used in the different steps of the synthesis has been boiled and then cooled under N_2 bubbling in order to avoid the dissolution of CO_2 from the atmosphere. Briefly, an aqueous solution of the phosphate and carbonate precursors is prepared by mixing a solution of $(NH_4)_2PO_4$ (300 mM) and a solution of $NaHCO_3$. The pH was adjusted to 10 by the addition of aqueous NH_3 (30 wt.%). This mixed solution was added to a solution of $Ca(NO_3)_2.4H_2O$ (100 mL; 500 mM) at 3 mL/min by an automatic titrator (Titrando 808, Metrohm). The mixture was stirred during 24h. The resulting particles were recovered by centrifugation (6000 rpm, 10 min) and then dried at 105°C for 3 days and heated at 400°C for 3 hours before characterization. The concentrations of the $NaHCO_3$ solution were 75, 150 and 300 mM and lead to an incorporation of 3.6, 5.2 and 8.1 w% of carbonate ions, respectively, as determined by elemental analysis. The powders are noted cHA-3.6, cHA-5.2, cHA-8.1, respectively. Without the addition of the $NaHCO_3$ solution and despite all the precaution used to avoid the dissolution of CO_2, a tiny amount of CO_3^{2-} is determined by elemental analysis (<1 w %). The corresponding powder is noted HA. The concentrations used for the synthesis are summarized in Table S2.

Bone samples preparation:

Bone samples were harvested from healthy 2 years old French ewes. Bone was extracted from the proximal part of the diaphysis and distal epiphysis of humerus and femur. The project 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. The NMR study was performed on fresh intact bone within 2 h after extraction (without any treatment) to prevent uncontrolled dehydration of the sample and to prevent any alteration by chemical pre-treatment. [2,4,7,8]

Analysis:

Solid State NMR. ^1H and ^{31}P solid state NMR experiments were recorded on a Avance 300 Bruker spectrometer operating at (^1H) = 300.13 MHz and (^{31}P) = 121.5 MHz. Dry powders (HA, cHA-3.6, cHA-5.2, cHA-8.1) were packed in 4 mm (O.D.) zirconia rotors and spun at 14 kHz. Wet

powders (CHA and CHA-SBF) were packed and placed in-between two Teflon spacers into a 4 mm (O.D.) zirconia rotor. They were wetted with 10 µL of double distilled water and then spun at 8 kHz. Wet samples (fresh bone, Coll/CHA, Coll/CHA(SBF)) were placed in-between two Teflon spacers into a 4 mm (O.D.) zirconia rotors and spun at 8 kHz. The NMR study was performed within 2 hours after extraction from untreated bone. $t_{90°}(^{1}H)$ and $t_{90°}(^{31}P)$ were 4.5 µs and 5.5 µs, respectively. ^{1}H and ^{31}P chemical shift was referenced (δ = 0 ppm) to TMS and 85 wt.% aqueous H_3PO_4, respectively. ^{31}P quantitative MAS experiments were single pulse experiments (recycle delay RD = 200 s). ^{1}H-^{31}P HetCor experiments parameters were the followings: (*i*) CHA, CHA-SBF, fresh bone, Coll/CHA and Coll/CHA(SBF): RD = 3.5 s, contact time CT = 10 ms, 400 transients for each 128 t_1 increments; (*ii*) HA, cHA-3.6, cHA-5.2, cHA-8.1: RD = 2 s, contact time CT = 1 ms, 232 transients for each 128 t_1 increments. The ^{31}P-filtered ^{1}H spectra were recorded through a $\{^{1}H$-$^{31}P\}^{1}H$ Double CP MAS experiment with a first $CT_1 = CT_2$ = 10-15 ms in order to maximize the hydroxyls resonance.

Powder X-ray diffraction (XRD) diagrams of all samples were performed on 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 5-57° range (2θ) with steps of 0.01° and a counting time of 9 s. The 9-432 JCPDS file was used to identify hydroxyapatite.

For TEM studies, the apatite samples were dispersed in ethanol. 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 hybrid matrices (Coll/CHA and Coll/CHA(SBF)), 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 samples were directly dehydrated (no staining) 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 (without staining).

Elemental analyses were performed by the Service Central d'Analyse of CNRS, Villeurbanne, France (Ca and P: ICP AES, ICAP 6300 from Thermofisher Scientific ; C: home-made microanalysor).

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.

Concentration (mM)	$CaCl_2$ $2H_2O$	NaH_2PO_4	K_2HPO_4	$NaHCO_3$	NaCl	KCl	$MgCl_2$ $6H_2O$	Na_2SO_4	$(CH_2OH)_3$ CNH_2	Ionic strength (mM)
CHA solution*	46.1	13.8	-	13.8	-	-	-	-	-	165.9
SBF solution*	2.5	-	1	4.2	142	3	1.5	0.5	-	165.7
SBF 1.5 solution (pH=7.4)	3.8	-	1.5	6.3	213	4.5	2.3	0.75	10	248.9

Table S1. Concentration of salt precursors used in mineralized collagen matrices syntheses. *The solutions were prepared in 500 mM acetic acid.

HA	500 mM $Ca(NO_3)_2$ $4H_2O$; 300 mM $(NH_4)_2HPO_4$
cHA-3.6	500 mM $Ca(NO_3)_2$ $4H_2O$; 300 mM $(NH_4)_2HPO_4$; 75 mM $NaHCO_3$
cHA-5.2	500 mM $Ca(NO_3)_2$ $4H_2O$; 300 mM $(NH_4)_2HPO_4$; 150 mM $NaHCO_3$
cHA-8.1	500 mM $Ca(NO_3)_2$ $4H_2O$; 300 mM $(NH_4)_2HPO_4$; 300 mM $NaHCO_3$

Table S2: Concentration of salt precursors for the synthesis of HA, cHA-3.6, cHA-5.2 and cHA-8.1

Sample	*a* (Å)	*c* (Å)
HA	9.425 ± 0.002	6.878 ± 0.001
cHA-3.6	9.430 ± 0.001	6.885 ± 0.001
cHA-5.2	9.420 ± 0.001	6.897 ± 0.001
cHA-8.1	9.414 ± 0.003	6.909 ± 0.002

Crystallite size	*a b* (nm)	*c* (nm)
HA	25	62
cHA-3.6	20	30
cHA-5.2	12	19
cHA-8.1	7	10

Table S3: Lattice parameters and crystallite size of HA, cHA-3.6, cHA-5.2 and cHA-8.1.

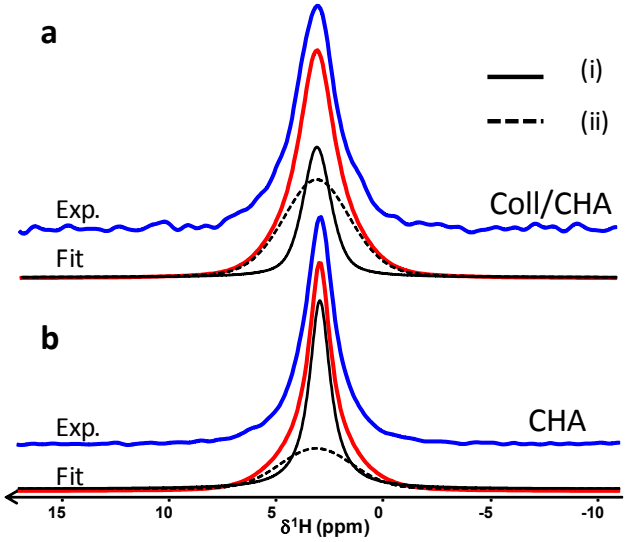

Figure S1 : a) and b) quantitative ^{31}P MAS spectra (blue) of Coll/CHA and CHA and the corresponding fitting (red) starting from the line shapes extracted from the 2D ^{1}H-^{31}P HetCor spectra (Fig. 1d & 1e) and corresponding to apatitic phosphates (i) and disordered phosphates (ii).

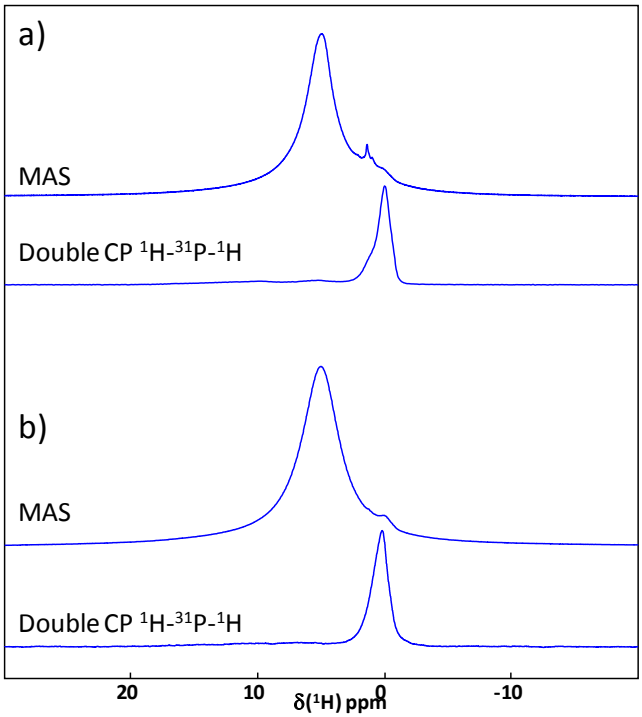

Figure S2: ^{1}H quantitative MAS and {^{1}H-^{31}P}^{1}H Double CP spectra of a) dry bone (CT_1 = CT_2 = 15 ms) and b) CHA-SBF (CT_1 = CT_2 = 10 ms). The {^{1}H-^{31}P}^{1}H Double CP allows the spectral edition of the apatitic OH^- ion resonance both for bone and CHA-SBF whereas the ^{1}H MAS spectra are blurred by unwanted ^{1}H signals from adsorbed water and extra cellular matrix (including collagen and non collageneous proteins) in case of bone.

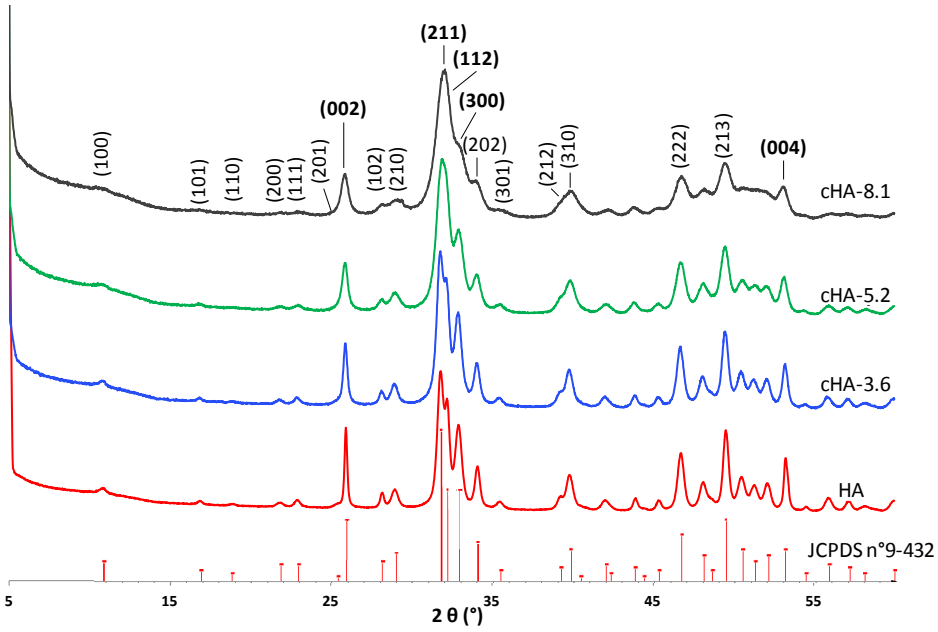

Figure S3 : XRD patterns of HA, cHA-3.6, cHA-5.2 and cHA-8.1

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