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Collagen Suprafibrillar Confinement Drives the Activity of Acidic Calcium-Binding Polymers on Apatite Mineralization

J r mie Silvent, Marc Robin, Camila Bussola Tovani, Yan Wang, Fabrice Soncin, Sidney Delgado, Thierry Azais, Capucine Sasso e, Marie-Madeleine Giraud-Guille, Jean-Yves Sire, and Nadine Nassif*



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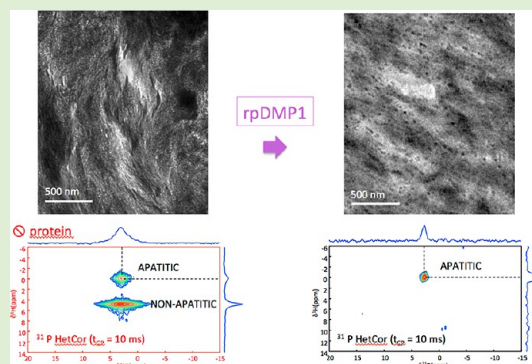


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Supporting Information

ABSTRACT: Bone collagenous extracellular matrix provides a confined environment into which apatite crystals form. This biomineralization process is related to a cascade of events partly controlled by noncollagenous proteins. Although overlooked in bone models, concentration and physical environment influence their activities. Here, we show that collagen suprafibrillar confinement in bone comprising intra- and interfibrillar spaces drives the activity of biomimetic acidic calcium-binding polymers on apatite mineralization. The difference in mineralization between an entrapping dentin matrix protein-1 (DMP1) recombinant peptide (rpDMP1) and the synthetic polyaspartate validates the specificity of the 57-KD fragment of DMP1 in the regulation of mineralization, but strikingly without phosphorylation. We show that all the identified functions of rpDMP1 are dedicated to preclude pathological mineralization. Interestingly, transient apatite phases are only found using a high nonphysiological concentration of additives. The possibility to combine biomimetic concentration of both collagen and additives ensures specific chemical interactions and offers perspectives for understanding the role of bone components in mineralization.



INTRODUCTION

Bone is a complex composite material, which closely associates cells and an extracellular matrix (ECM). The bone ECM combines various components, that is, collagen, noncollagenous proteins (NCPs), carbonated hydroxyapatite (HA) nanoparticles, and water. Although bone is one of the most studied living materials, the exact role of its components, noticeably the organic one, is still debated. Both intra-^{1–3} (~40 nm) and interfibrillar⁴ (~1.5 nm) spaces in collagen are proposed to be nucleation sites for bone mineral. The interfibrillar confinement generated by the collagen matrix increases as the ECM becomes mineralized, thus forming the mature bone (Figure 1A) with the characteristic twisted plywood pattern⁵ (Figure 1B). This suprafibrillar three-dimensional (3D) architecture affects the physical properties of the ECM such as mechanical response^{6,7} and microenvironment (e.g., size and spatial distribution of apatite, local hydrated environment of phosphate ions).⁸ In addition, *in vitro* investigations on NCPs activity have indeed yielded contradictory findings when the protein is studied either in solution or in gel.^{9,10} Upon working with gels, loose collagen matrices imply large interfibrillar spaces due to the lack of collagen packing,⁸ meaning that bone is only reproduced at the fibril level.^{11,12} Consequently, aside *in vivo*-based experiments, biomimetic models in terms of confinement are needed to ensure that investigations of NCPs activity are conducted

under native environment. As demonstration, only models mimicking both collagen density and geometry described for mature bone (250 mg/mL)⁸ tissues led to the typical apatite/collagen co-alignment (Figure 1C), but noticeably with higher concentrations of calcium ions (Figure 1D, left) than that described in extracellular fluid (ECF).¹³ Indeed, simulated body fluid (SBF) failed to produce intrafibrillar mineral; instead, apatite in the form of spherulite was found (Figure 1D, right). This result suggested that Ca-rich proteins from bone ECM concentrate ions in the gap regions.

To go further on the effect of bone ECM confinement on NCPs activity, we pursue the study by entrapping dentin matrix protein-1 (DMP1), an acidic calcium-binding polymer¹⁴ within our models. DMP1 is a phosphorylated ECM protein produced by osteocytes and odontoblasts that is commonly accepted as mediator in bone mineralization processes.^{15–17} Indeed, DMP1 knockout mice display defective mineralization of dentin^{18–21} and bone^{22,23} resembling phenotypes observed in human genetics. Interestingly, the 65

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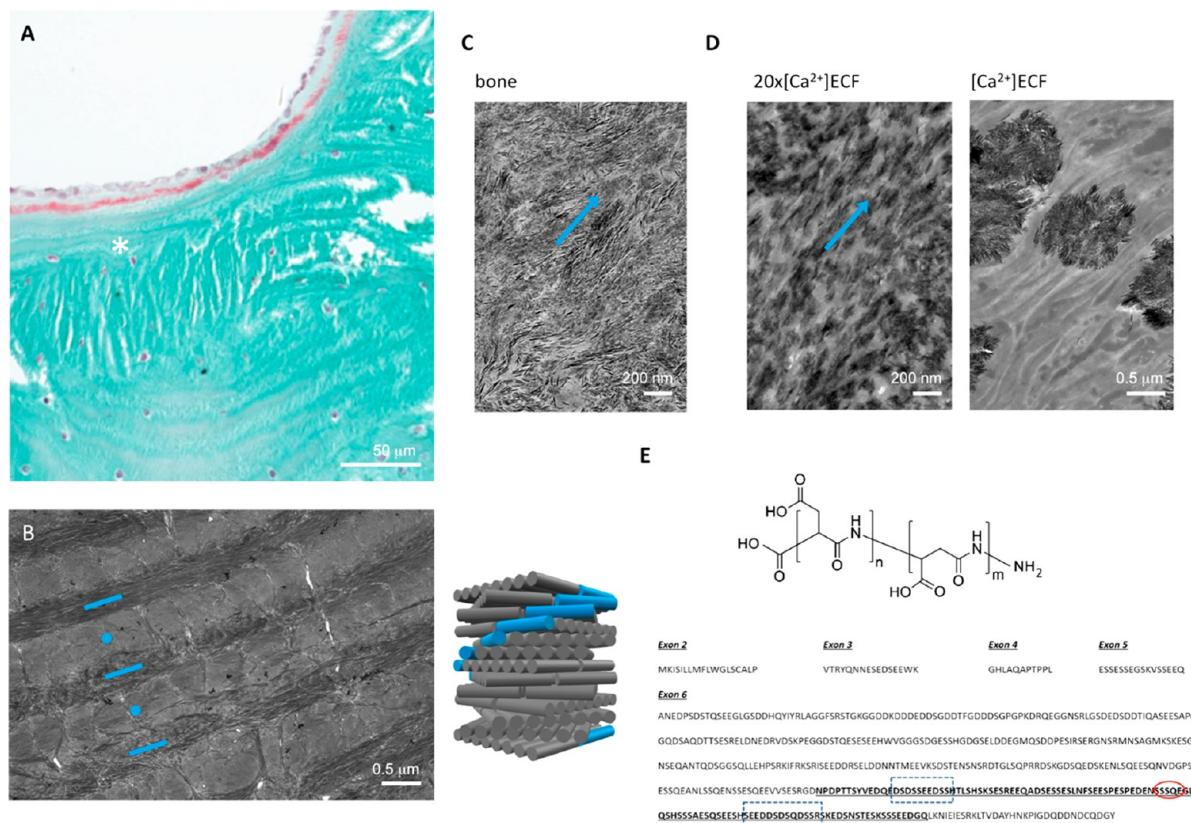


Figure 1. Comparison of collagen/apatite relationship in bone and in synthetic models reproducing the collagen confinement (density and order) found in bone. (A) Thin section of bone stained with Masson's trichrome. From top to bottom: Osteoblasts, osteoid tissue (light blue and red), and mature bone (*). Transmission electron microscopy ultrathin sections of (B) mature decalcified human bone that is characterized by a twisted plywood organization of collagen fibrils (blue dot and bars represent molecules that are perpendicular and parallel to the observation plan, respectively) schematically represented, (C) unstained sheep bone where the alignment of apatite platelets along the fibril is observed (the blue arrow shows the orientation), (D) Coll/CHA/SBF (left) and Coll/SBF/SBF (right) where $[\text{Ca}^{2+}] = 20[\text{Ca}^{2+}]_{\text{ECF}}$ and $[\text{Ca}^{2+}] = [\text{Ca}^{2+}]_{\text{ECF}}$, respectively. The collagen/apatite co-alignment similar to that in bone is observed in Coll/CHA/SBF (left) while spherulitic apatite crystals are obtained in Coll/SBF/SBF (right). In (E), the acidic calcium-polymer used in the biomimetic model, that is, the polyaspartate formula and the human DMP1 sequence (in bold: target peptide; blue boxed texts: collagen binding sites; red encircled text: motif found as unchanged during 220 My). The influence of organic polymers on apatite formation is studied at fixed physiological concentration of calcium, that is, $[\text{Ca}^{2+}] = [\text{Ca}^{2+}]_{\text{ECF}}$ with two different concentrations for rpDMP1 as follows: 2.5 $\mu\text{g}/\text{mL}$ (1rpDMP1) and 25 $\mu\text{g}/\text{mL}$ (10rpDMP1).

66 role of DMP1 has been investigated *in vitro* through different
 67 models (e.g., in solution,^{24,25} adsorbed onto glass plates,^{14,26} in
 68 gelatin-gel systems,^{15,27} on a transmission electron microscopy
 69 (TEM) grid coated with recombinant spider silks,²⁸ with
 70 preassembled collagen fibrils,^{29,30} and with early calcifying
 71 bone-like matrix),³¹ reaching the conclusion that the protein
 72 could be involved at different levels including in apatite
 73 nucleation control^{27,32} and growth inhibition,³³ in mediating
 74 the size of collagen fibrils,²⁹ and in stabilizing amorphous
 75 calcium phosphate (ACP) phase.^{33,34} To clarify such func-
 76 tional diversity, a DMP1 recombinant peptide (rpDMP1)
 77 containing those two collagen binding sites²⁹ and the peptide
 78 ⁴²⁷SSSQE⁴³¹ was synthesized and used here (unphosphory-
 79 lated, 121 amino acids, 13 kDa, pI = 4.14) (Figure 1E, down).
 80 Indeed, four DMP1 peptides are identified in dentin and bone
 81 extracts: (1) the full-length protein, (2) a N-ter fragment of 37
 82 kDa, (3) a C-ter fragment of 57 kDa,³⁵ and (4) a proteoglycan
 83 derived from the N-ter fragment and known as DMP1-PG
 84 found in rat and mouse.³⁶ Importantly, five highly conserved
 85 motifs during mammalian evolution were identified including
 86 ⁴²⁷SSSQE⁴³¹, suggesting the importance of this acidic peptide
 87 for the protein structure and/or function.³⁷ Moreover, it was
 88 reported that the 57 kDa fragment recapitulates the function of

full-length DMP1 in regulation of mineralization and osteocyte
 89 maturation.³⁸ Finally, phosphorylation appears to be dedicated
 90 to the organization of mineral when there is no collagen
 91 fibrillar packing³⁹ since both phosphorylated and non-
 92 phosphorylated rDMP1 are proposed as apatite nucleators.⁴⁰ 93

In addition to the confinement criterion, the concentration
 94 of protein closer to physiological conditions^{41,42} is of
 95 importance to access to their native properties. Noticeably,
 96 differences in range of concentrations (factor 10 to 100) can
 97 lead to opposite activities^{14,15,24,26,29} blurring the conclusions. 98

To validate the specificity of the 57-KD fragment of DMP1,
 99 experiments were also carried out with a synthetic calcium-
 100 binding polymer, that is, the biomimetic poly-L-aspartic acid
 101 (polyAsp, 1.2 kDa, pI = 2.98) (Figure 1E, up).^{43,44} Nowadays,
 102 polyAsp is commonly used in biomineralization models for
 103 mimicking acidic proteins in biological calcified tissues (nacre,
 104 bone, dentin). This polymer is described to allow the
 105 intrafibrillar infiltration of both DMP1⁴⁵ and apatite ion
 106 precursors⁴⁶ and consequently the collagen/mineral co-align-
 107 ment.⁴⁷ 108

Here, we show that collagen suprafibrillar confinement
 109 drives the activity of acidic calcium-binding polymers on apatite
 110 mineralization. The difference in mineralization between 111

112 rpDMP1 and polyAsp illustrates the specificity of NCPs amino
 113 acids sequence. Under confinement (i.e., intra- and interfi-
 114 brillar spaces), we show that all the identified functions of
 115 rpDMP1 are dedicated for proper calcification to occur,
 116 namely the collagen/apatite co-alignment.^{48,49} Indeed, we
 117 show that while collagen nucleates apatite, rpDMP1 (i)
 118 concentrates the apatite ion precursors locally, interfering as
 119 a supporting agent for collagen (ii) to induce the formation of
 120 a first highly crystalline apatite crystal. In addition, we discard
 121 that rpDMP1 stabilizes the possible transient precursors of
 122 bioapatite^{50,51} since they only form at high nonphysiological
 123 concentrations of protein. Finally, (iii) we confirm that it
 124 inhibits the apatite growth, but (iv) also show that it might
 125 inhibit the homogeneous nucleation irrespective of confine-
 126 ment. Overall, the work demonstrates that aside from cellular
 127 and biochemical processes, physicochemical parameters take
 128 part in the control of bone biomineralization.

129 ■ MATERIALS AND METHODS

130 **Synthesis and Purification of the Recombinant DMP1**
 131 **Peptide. Cloning.** The coding sequence of our targeted human
 132 DMP1 peptide (i.e., amino acids 367–481) was isolated from a
 133 human primary osteoblast cDNA (Promocell). It was amplified in a
 134 thermal cycler (Mastercycler pro, Eppendorf) by PCR using the
 135 oligonucleotides 5' ATGC CATATG AACCCCGACCCCA 3' and 5'
 136 GCAT CTCGAGTCA GTG GTG GTG GTG GTG GTG GTG
 137 CAACTGGCCATCTTC 3' to create NdeI and XhoI restriction sites
 138 at the start and stop codon, respectively. Cycling conditions were
 139 initial denaturation at 94 °C for 2 min, followed by 35 cycles, each
 140 cycle consisting of 30 s of denaturation at 94 °C, 45 s of annealing at
 141 60 °C and 45 s of elongation at 72 °C. The final elongation lasted for
 142 2 min at 72 °C. PCR products were analyzed by 1% agarose gel
 143 electrophoresis and observed in an analyzer Gel Doc (BIORAD,
 144 France) after ethidium bromide staining. The amplified fragment was
 145 purified using the QIAquick PCR purification kit (Qiagen SA) and
 146 digested with NdeI and XhoI restriction enzymes. The resulting
 147 products were separated by a migration in a 1.5% agarose gel with
 148 ethidium bromide and cloned into the pET22b vector (Novagen/
 149 VWR International S.A.S) which had been beforehand digested
 150 similarly. Competent Novablue cells were transformed with the
 151 ligation mix and selected by overnight growth on LB agarose plates
 152 containing 70 µg/mL ampicillin. Positive clones were checked for the
 153 presence of the 0.8 kb fragment by NdeI and XhoI restriction analysis.
 154 **Expression.** BL21 (DE3) cells transformed with pET-DMP1 were
 155 grown on a rotating table (220 rpm) overnight at 37 °C in LB
 156 medium with 50 µM ampicillin. When the optical density at 600 nm of
 157 the bacterial broth reached 0.6–0.8, the induction was realized with
 158 an addition of 1 mM of isopropyl β-D-1-thiogalactopyranoside. After 3
 159 h, the cells were centrifuged at 5000g for 10 min, and the pellets were
 160 frozen at –20 °C until purification.
 161 **Purification.** Pellets were resuspended in 5 mL of buffer A (PBS
 162 1×, 50 mM imidazole, complete inhibitor mix (Roche), lysozyme,
 163 DNase) and lysed by sonication three times. The bacterial extract was
 164 loaded on a 5 mL bed-volume HisTrap column using an Akta Purifier
 165 10 (GE-Healthcare). Buffer A was flowed through the column at 1
 166 mL/min until A₂₈₀ of the flow through reached a stable value. A 60
 167 mL linear gradient of 0–100% Buffer B (PBS 1×, 500 mM imidazole,
 168 complete inhibitor mix (Roche), lysozyme, DNase) in buffer A was
 169 applied, and 1 mL fractions were collected. Aliquots were analyzed by
 170 10% SDS-PAGE and the gels stained with Coomassie blue to
 171 determine the quality of purified rpDMP1 (12 kDa, pI = 4.14)
 172 (Figure S6).

173 **Sample Preparation. Collagen Extraction.** A solution of type I
 174 collagen at ~3 mg/mL in 0.5 M acetic acid was prepared as previously
 175 described.⁵² The collagen was extracted from rat tail tendons. After a
 176 washing step with PBS, tendons were solubilized in 0.5 M acetic acid,
 177 and the solution was clarified by centrifugation (21,000 rpm, 2 h, 11
 178 °C). The supernatant was selectively precipitated with 0.3 and 0.6 M

of NaCl by two centrifugations (21,000 rpm, 3 h, 11 °C then 4400
 rpm, 45 min, 11 °C), in order to remove proteins other than type I
 collagen and collagen, respectively. The pellets were solubilized in 0.5
 M acetic acid and dialyzed against 0.1 M acetic acid in order to
 remove salts from the solution. A final centrifugation was performed
 (21,000 rpm, 4 h, 11 °C), and the concentration was adjusted to a
 final stock concentration of ~3 mg/mL. The final concentration of
 type I collagen solution was estimated by hydroxyproline titration.⁵³

Synthesis of Collagen/Apatite Matrices. The matrices were
 prepared according to a procedure that combines injection and
 reverse dialysis processes (patent WO2011151587A2). The mineral-
 ization conditions for collagen matrices (concentrations of compo-
 nents) are summarized in Table 1. Collagen samples were disk-shaped

Table 1. Mineralization Conditions for Collagen Matrices⁴⁴

sample designation	end collagen concentration (mg/mL)	acidic polymer (µg/mL)	HA ion precursors in dialysis solution (acetic acid)	additional SBF bath
Coll	250	–	–	–
Coll/SBF	250	–	SBF	–
Coll/SBF/SBF	250	–	SBF	+
Coll/CHA/SBF	250	–	CHA	+
Coll/SBF(rpDMP1)	250	2.5	SBF	–
Coll/SBF(rpDMP1)/SBF	250	2.5	SBF	+
Coll/10rpDMP1	250	25	–	–
Coll/SBF(10rpDMP1)	250	25	SBF	–
Coll/SBF(10rpDMP1)/SBF	250	25	SBF	+
Coll/SBF(polyAsp)	250	5.75	SBF	–
Coll/SBF(polyAsp)/SBF	250	5.75	SBF	+
CollOsteoid/SBF/SBF	40	–	SBF	+
CollOsteoid/SBF(10rpDMP1)/SBF	40	25	SBF	+

⁴⁴Ionic composition of SBF and CHA solutions are detailed in Table S1.

with a thickness of ~1 mm and a diameter of ~10 mm. A soluble
 acidic collagen solution (1 mg/mL, 0.5 M acetic acid) supplemented
 by a 1× SBF solution mimicking the ionic compounds found in the
 human plasma was prepared by diluting the stock solution (3 mg/mL,
 0.5 M acetic acid) with a 1.5× SBF acidic solution (0.5 M acetic acid).
 SBF was prepared as previously described.⁵⁴ The concentrations of
 the salts precursors are summarized in Table S1. Two concentrations
 of rpDMP1 were added to this solution: 2.5 µg/mL (low) and 25 µg/
 mL (high) to form the matrices referred as Coll/SBF-rpDMP1 and
 Coll/SBF-10rpDMP1, respectively. The rpDMP1 control matrix with
 the highest concentration of rpDMP1 and without mineral (Coll/
 10rpDMP1) was obtained by diluting the stock solution with acetic
 acid (0.5 M). Two matrices used as control were prepared: (i)
 without any organo-mineral additives (Coll) and (ii) with apatite ion
 precursors but without addition of any acidic polymer (Coll/SBF).
 The matrices supplemented by polyaspartate (Coll/SBF-polyAsp)
 were obtained in a similar way, by adding this acidic polymer (5.75
 µg/mL, Lanxess-Bayer, BaypureDS100, 1200 g/mol, pI = 2.98) to the
 acidic collagen solution. To mimic the osteoid tissue⁵⁵ CollOsteoid/
 SBF/SBF, 40 mg/mL collagen matrices were also formed. In this case,
 25 µg/mL rpDMP1 was added to the acidic collagen solution forming
 the matrix referred as CollOsteoid/SBF-10rpDMP1/SBF. All these
 solutions were continually injected in a closed dialysis chamber for 1
 week. The bottom of the chamber contained a dialysis membrane
 with a molecular weight cut off of 1 kDa. The reverse dialysis
 process⁵⁶ was set against polyethylene glycol (PEG, 35 kDa, Fluka) to

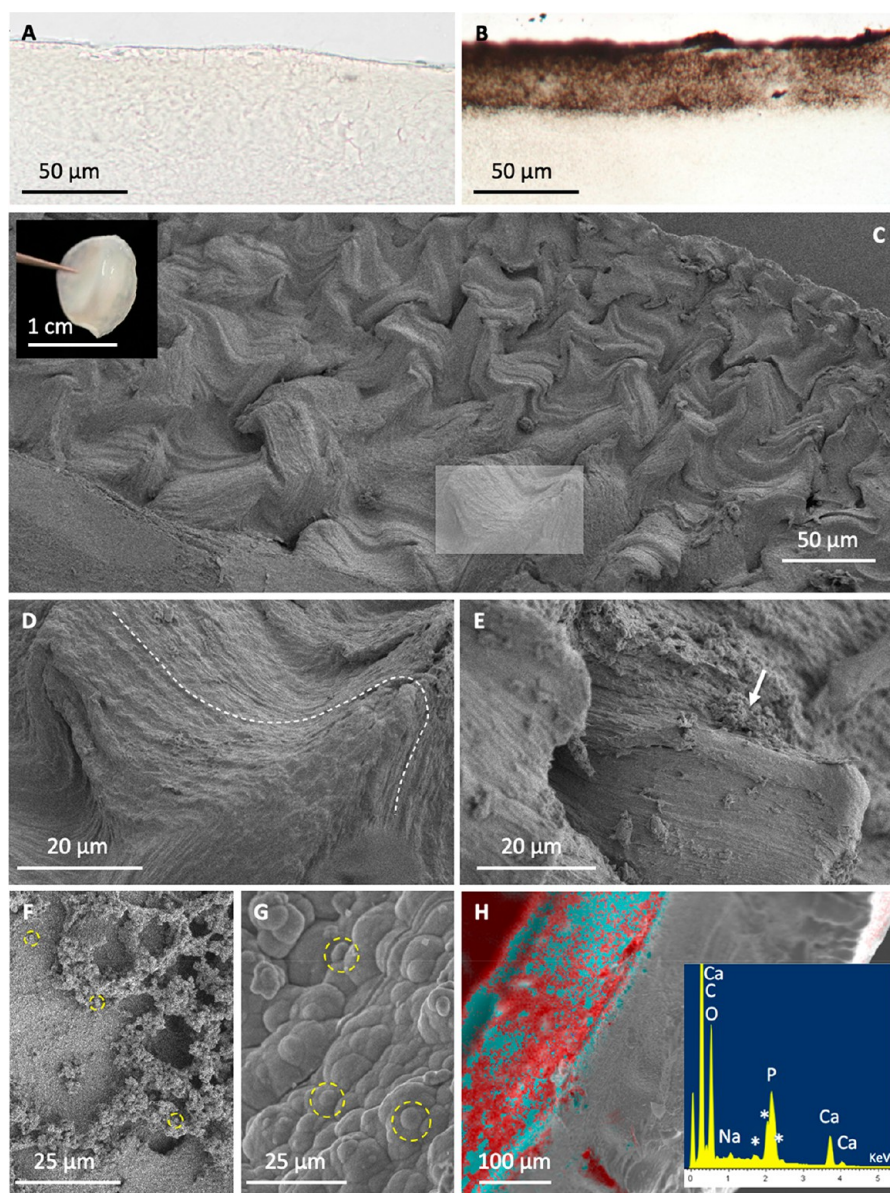


Figure 2. Structural characterizations of the hybrid collagen/apatite matrices in the presence of rpDMP1 containing the highly conserved motif. Histological sections of Coll/SBF(1rpDMP1) (A) and Coll/SBF(1rpDMP1)/SBF (B) stained with von Kossa. SEM observations of the dense collagen matrix (250 mg/mL) at low rpDMP1 content (C–F). Examination of the fractured interior of the disk-shaped matrix (C, inset) before SBF bath (Coll/SBF(1rpDMP1)) (C, D; rectangle in C indicates the enlarged section that is shown in D). Examination after SBF bath (Coll/SBF(1rpDMP1)/SBF): interior (E) and surface (F). In (E), the roughness appears to increase after SBF (arrow). (G) Surface of the collagen matrix precipitated without rpDMP1 (Coll/SBF/SBF). The size and the amount of spherulitic crystals (yellow dashed circles) at the surface of the matrices decrease with rpDMP1. (H) Analysis of Coll/SBF(1rpDMP1)/SBF by EDX coupled with SEM; calcium ions are in red, and phosphorus ions in blue (SEM gold coating*).

218 control the final concentration of collagen. The PEG was dissolved in
219 0.5 M acetic acid up to ~300 mg/mL for all Coll matrices or ~50
220 mg/mL for all CollOsteoid matrices. To form the mineralized
221 matrices, the ionic precursors of SBF or CHA were dissolved in the
222 PEG/acetic acid solution. After injection of the total amount of
223 collagen, dialysis was continued for 1 week in order to obtain a
224 homogeneous concentration in the samples and a good maturation of
225 the peptides. The pH was then increased to a range of 9–10 by
226 ammonia gas diffusion for 3 days to induce collagen fibrillogenesis,
227 stabilize the liquid crystalline organization into dense fibrillar
228 matrices, and precipitate the mineral phase. These matrices were
229 then removed from the dialysis chamber and washed several times in
230 sterile double-distilled water until complete neutralization. The final
231 concentration of type I collagen in the different collagen matrices was
232 assessed by hydroxyproline titration and found to be ~250 mg/mL

for all Coll matrices and ~40 mg/mL for all CollOsteoid matrices. 233
The different matrices were split into halves, and the mineralization 234
degree of one-half was increased by a bath in 45 mL of 1.5X SBF 235
solution at pH = 7.4 under mild rotary stirring (220 rpm) at 37 °C for 236
1 week.⁸ 237

Mineral Characterization. Histology. Bone samples were fixed in 238
4% paraformaldehyde and embedded in paraffin for sectioning. Ten 239
µm-thick serial sections perpendicular to the cell layer were dewaxed, 240
rehydrated, and stained specifically by von Kossa, thus identifying 241
divalent ions. The slides were rinsed, dehydrated, and mounted for 242
observation with an optical microscope (Nikon E600 POL) or an 243
epifluorescence microscope (AXIO 100 Zeiss). 244

**Scanning Electron Microscopy and Energy-Dispersive X-ray 245
Spectroscopy.** Each sample was fixed in 3.6% glutaraldehyde in a 246
cacodylate/saccharose buffer solution (0.05 M/0.6 M -pH 7.4). The 247

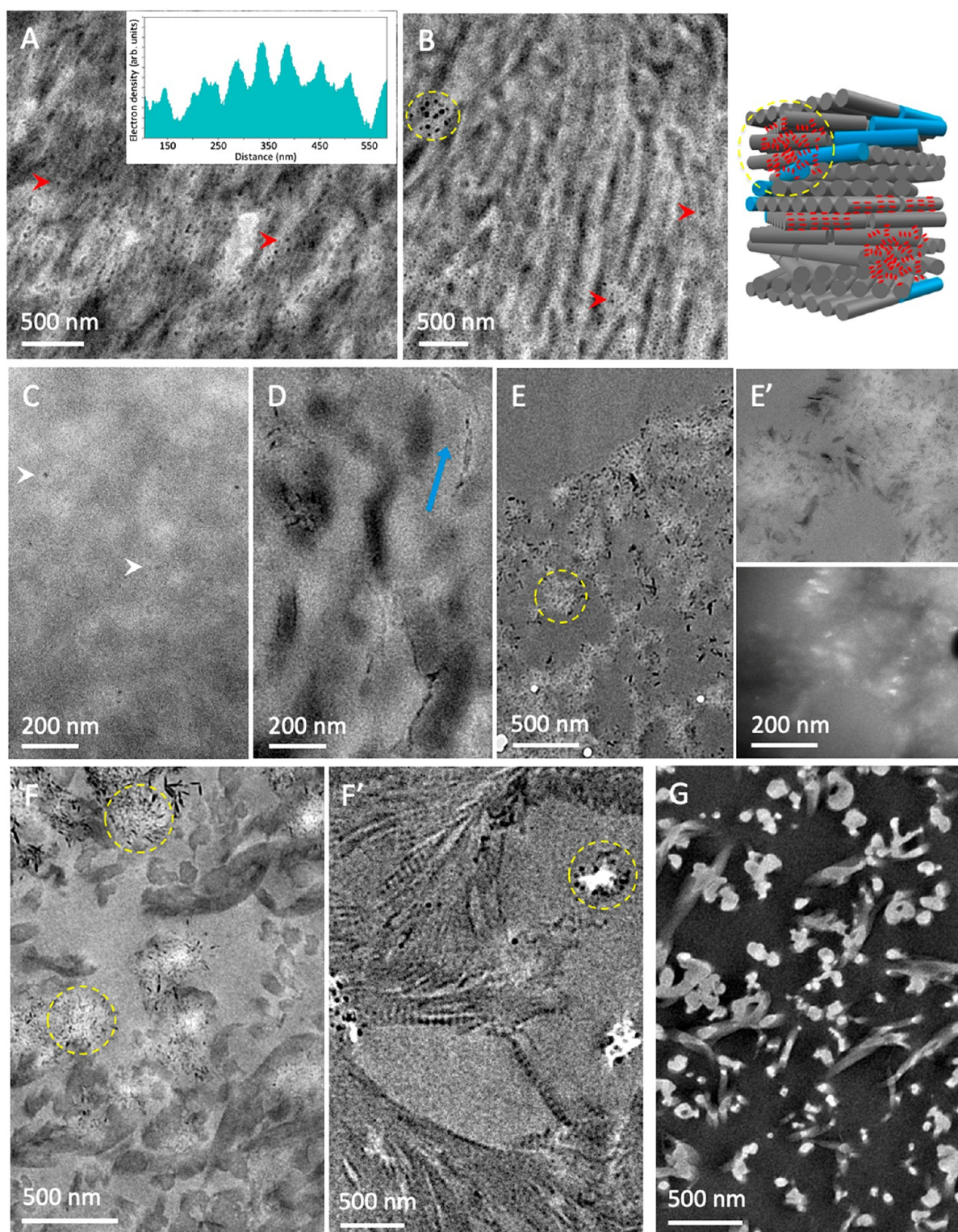


Figure 3. Investigations by TEM of calcium phosphate precipitation in rpDMP1 containing a mature bone-like matrix. Observations of unstained ultrathin sections of (A) Coll/SBF(1rpDMP1) and (B) Coll/SBF(10rpDMP1) before the SBF bath. Both matrices present nanometric electron-dense precipitates (red arrows) along and/or within the collagen fibrils with periodicity highlighted by the intensity profile. In addition, aggregates of the “dot-like” precipitates are seen in Coll/SBF(10rpDMP1); the scheme illustrates the corresponding distribution of dot-like precipitates (red) related to the organization of collagen fibrils. They are found along the fibrils or as aggregates (yellow dashed circle). (C) A control matrix prepared with the high concentration of rpDMP1 (25 $\mu\text{g}/\text{mL}$) and without ionic apatite precursors (Coll/10rpDMP1) shows that collagen fibrils are hardly observed in contrast to the precipitated proteins (yellow arrows). After immersion in SBF, (D) axial alignment of platelets are observed inside the matrix. (E) Observations within the most superficial layer of the matrix show the presence of spherulitic crystals (E') according to bright-/dark-field images. Observations at the surface of Coll/SBF(10rpDMP1)/SBF (F) without and (F') with staining where the dashed yellow circles highlight the presence of spherulite. Observations at the surface of (G) Coll without staining.

248 samples were dehydrated through successive ethanol bath (50%, 70%,
249 80%, 90%, and 100%), and a supercritical CO₂ drying process was
250 performed on a BAL-TEC 030. Then samples were sputter-coated
251 with a gold layer of 10 nm and observed in a Hitachi S-3400N
252 operating at 3 kV.

253 Energy-dispersive X-ray (EDX) microanalysis was used for the
254 mapping of mineral deposits inside the matrices. The EDX instrument
255 X-Max (Oxford Instruments) was coupled to a scanning electron
256 microscope Hitachi S-3400N operating at 12 kV, and the Oxford
257 Microanalysis Group XAN.70 software was used for this analysis.

258 **Transmission electron microscopy.** This protocol is similar to the
259 protocol for the scanning electron microscopy (SEM). Then, samples
260 were rinsed, dehydrated, and embedded in Epon 812. For a few
261 samples (always mentioned in the text), an additional postfixation was
262 carried in 2% osmium tetroxide in a cacodylate/saccharose buffer
263 solution (0.4 M/0.6 M-pH 7.4) during 1 h at 4 °C, otherwise neither
264 osmium nor uranyl acetate (staining) were added to avoid artifacts on
265 ultrathin sections. Sections (~80 nm) were observed with a Tecnai
266 spirit G2 operating at 120 kV.

267 **Wide-Angle X-ray Diffraction (Transmission Mode).** Matrices
268 were inserted in X-ray cylindrical borosilicate capillary tubes. The
269 tubes were wax-sealed to keep the samples hydrated and placed
270 directly in the vacuum chamber beam. X-ray diffraction experiments
271 were performed with a S-MAX 3000 RIGAKU using a mono-
272 chromatic CuK α radiation. The diameter of the cylindrical beam
273 dimension of the specimen was 400 μ m. The data were collected in
274 the 3–60° range (2θ). The sample-to-detection distance was 0.059 m
275 with a diameter of capillary tubes at 1 mm or 0.058 m with a diameter
276 of 2 mm. The two-dimensional (2D) wide-angle X-ray diffraction
277 (WAXD) patterns were collected with imaging plates then scanned.
278 The data were analyzed using Image (LPS, U-psud) software.

279 **ζ -Potential Measurements.** The matrices were washed before
280 characterization precluding the involvement of free ions in the
281 resulting global charge. Samples were crushed in liquid nitrogen, and
282 the resulting powders were dispersed in PBS solution. Measurements
283 were carried out using a Malvern Zetasizer Nano ZS90.

284 **Thermogravimetric Analysis.** Samples were dried under a laminar
285 hood overnight to minimize the mass loss of loosely bounded water.
286 The analysis was performed on a thermo-microbalance instrument
287 (NETZSCH STA 409PC). The measurement was performed from room
288 temperature to 1000 °C in an oxidizing atmosphere (air) with a
289 heating rate of 5 °C/min.

290 **Nuclear Magnetic Resonance.** Solid-state nuclear magnetic
291 resonance (ssNMR) experiments were realized on hydrated samples.
292 Magic angle spinning (MAS) spectra were acquired at a frequency of
293 8 kHz, with samples packed into 4 mm zirconia rotors. ¹H–³¹P cross-
294 polarization (CP) experiments were performed on an Avance 300
295 Bruker spectrometer operating at frequencies of 300.13 MHz (¹H)
296 and 121.50 MHz (³¹P). The contact times (CT) were set at 10 and 1
297 ms, and the recycle delay (RD) at 2 s. A 2D ¹H–³¹P heteronuclear
298 correlation (HETCOR) was performed with the following param-
299 eters: RD = 2 s, CT = 10 and 1 ms, 1280 transients for each 128 t1
300 increments. ¹H and ³¹P chemical shifts were referenced ($\delta = 0$ ppm)
301 to adamantane and to 85%w aqueous H₃PO₄, respectively.

302 ■ RESULTS AND DISCUSSION

303 **Structural Characterizations of rpDMP1-Loaded Min-**
304 **eralized Collagen Matrices.** Experimental conditions and
305 matrices composition (see **Materials and Methods**) are
306 summarized in **Table 1**. It includes collagen matrices without
307 additives used as control, namely Coll, Coll/SBF (**Figure S1**),
308 Coll/CHA/SBF (**Figure 1D**, left), and Coll/SBF/SBF (**Figure**
309 **1D**, right).⁸ This means that after coprecipitation of collagen
310 with either CHA (~20 times more concentrated in apatite ion
311 precursors see **Table S1**) or SBF, these two last matrices were
312 then immersed in SBF to mimic further steps of mineral
313 growth. With additives (rpDMP1, polyAsp), fibril precipitation
314 was only performed in SBF ($[\text{Ca}^{2+}] = [\text{Ca}^{2+}]_{\text{ECF}}$)⁵⁷ to set

physiological-like conditions. Two concentrations of rpDMP1 315
were used here, for which we will refer to low (2.5 μ g/mL, 316
1rpDMP1) and high (25 μ g/mL, 10rpDMP1). For a better 317
reading, data for 10rpDMP1 are displayed in the main text 318
only when differences in mineral formation are observed 319
between the two concentrations. Additional data for 320
10rpDMP1 are shown in **Figure S2**. 321

We first investigated the spreading of mineral within 322
collagen matrices before and after immersion in SBF for 323
both rpDMP1 concentrations. For this purpose, we used von 324
Kossa staining on histological sections as it allows observations 325
at large scale. Interestingly, staining is hardly observed without 326
SBF extra-bath (Coll/SBF(1rpDMP1), **Figure 2A**), whereas a 327
strong staining is observed for both matrices at the superficial 328
layer (~50 μ m thick) after immersion (Coll/SBF(1rpDMP1)/ 329
SBF, **Figure 2B**). Because von Kossa staining may interfere 330
with any divalent ions,⁵⁸ further characterizations were 331
performed to confirm the presence of mineral. 332

To identify the distribution of the minerals at a lower scale, 333
investigations were performed at the surface and inside 334
fractured disk-shaped matrices (**Figure 2C–G**) using SEM 335
coupled with EDX microanalysis (**Figure 2H**). Observations 336
inside the matrix reveal that the entrapment of rpDMP1 does 337
not disturb the self-assembly of collagen fibrils since twisted 338
plywood structures form over large distances (**Figure 2C,D** 339
versus **Figure S1**, Coll/SBF). After immersion in SBF (Coll/ 340
SBF(1rpDMP1)/SBF), the surface roughness is more 341
pronounced by the deposition of particles on the closely 342
packed oriented fibrils (**Figure 2D** versus **2E**, arrow). On the 343
surface of the matrices, spherulites usually consisting of 344
aggregates crystals⁵⁹ are observed, and, interestingly, they 345
appear to be smaller in the presence of the protein (~0.5 μ m 346
versus ~4 μ m in Coll/SBF/SBF, yellow dashed circles in 347
Figure 2F,G). In addition, they appear less abundant since they 348
do not cover the whole surface. EDX analysis shows that the 349
spherulites are mainly composed of calcium and phosphorus 350
atoms with an average Ca/P ratio of about 1.66–1.71, 351
suggesting the formation of apatite in the presence of 352
rpDMP1 (**Figure 2H**).⁶⁰ 353

Confinement Effect on Apatite/rpDMP1 Distribution. 354
TEM investigations were performed on thin sections of Coll/ 355
SBF(1rpDMP1) and Coll/SBF(10rpDMP1) to access higher 356
magnifications. TEM sections were not stained to avoid the 357
presence of staining deposits that are difficult to distinguish 358
from CaP crystals.⁸ Observations evidence the presence of 359
nanometric electron-dense (“dot-like”) precipitates (~30 nm) 360
with low and high rpDMP1 concentrations (red arrows in 361
Figure 3A,B, respectively) within the dense collagen network at 362
this scale. The striated pattern (67 nm) is observed locally 363
(**Figure 3A**, inset), indicating that some minerals localize inside 364
the gap regions.^{8,61} Interestingly, additional aggregates of 365
nanoprecipitates are observed for high rpDMP1 content 366
(**Figure 3B**, yellow dashed circle). 367

To help identify the nature of the “dot-like” precipitates, a 368
control matrix was prepared without apatite ion precursors 369
(Coll/10rpDMP1) (**Figure 3C**). Likewise, “dot-like” particles 370
are observed (white arrows), confirming that the nano- 371
precipitates are also composed of the protein, in agreement 372
with previous observations.¹⁴ Although it is difficult to 373
conclude due to the low contrast of organic components in 374
TEM, the precipitates appear less abundant and not distributed 375
along the fibrils (**Figure 3C** versus **Figure 3A,B**). Nevertheless, 376
spherulites are observed without rpDMP1 in SBF (Coll/SBF/ 377

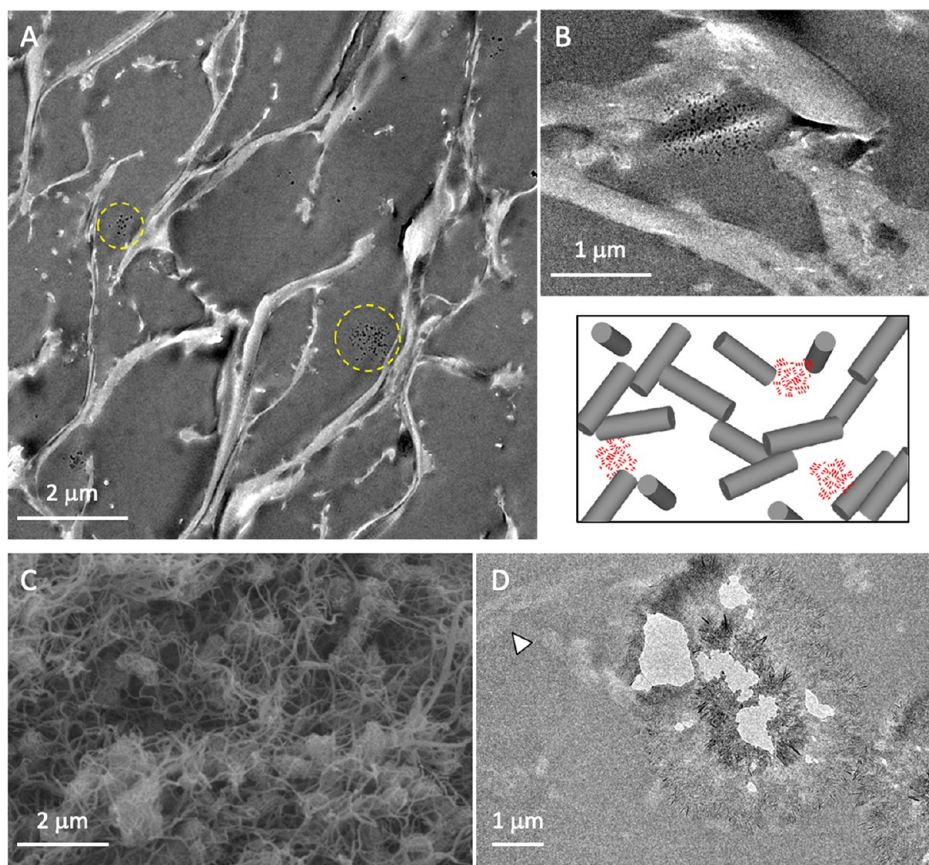


Figure 4. Investigations of calcium phosphate precipitation in rpDMP1 containing osteoid-like matrix. TEM observations of unstained ultrathin sections of Coll/Osteoid/SBF(10rpDMP1)/SBF (A) at low magnification where “dot-like” precipitates aggregate locally (yellow dashed circle). They are clearly seen (B) at higher magnification. Below, the corresponding relationship between mineral and collagen is represented in the scheme. (C) SEM and (D) TEM micrographs of Coll/Osteoid/SBF/SBF. The absence of staining makes the observation of cross-striated fibrils usually seen in a longitudinal cut (white arrow) difficult.

378 SBF in Figure 1D and Coll/SBF⁸), indicating that rpDMP1
379 may interfere with some ions to localize specifically inside the
380 gap zone.

381 After a SBF bath, few platelets with axial alignment (blue
382 arrow in Figure 3D) are observed for both rpDMP1
383 concentrations together with spherulitic particles at the surface
384 and within the most superficial layer of the matrix (dashed
385 yellow circles in Figure 3E,F). Noticeably, they appear smaller
386 in size (~300 nm versus ~2 μm) than those found without
387 rpDMP1 (Figure 1D, right), but remain crystalline according
388 to the contrast observed in the dark-field TEM image (Figure
389 3E'). Further observations at the surface of Coll/SBF-
390 (10rpDMP1)/SBF allow the visualization of both the resin
391 and individual fibril due to a lower density of collagen locally.
392 A comparison between mineralized collagen fibrils without or
393 with staining (Figure 3F,F', respectively) confirms the
394 precipitation of fibrils with the cross-striated pattern.
395 Observations of an unmineralized collagen matrix without
396 staining (Coll, Figure 3H), where the resin is darker than
397 collagen, confirm that the contrast in mineralized samples
398 comes from the spreading of mineral (ions or precipitates)
399 over the collagen fibrils. Note that we cannot conclude on the
400 involvement of DMP1 in mediating the size of fibrils,²⁹ since
401 the average diameter of collagen fibrils appears unmodified
402 (~100 nm) regardless the rpDMP1 concentration.

403 Going further, thermogravimetric analysis (TGA) inves-
404 tigations were performed to better characterize the effect of

rpDMP1 concentration on apatite formation (Figure S3). 405
Considering the standard deviation recorded for the matrices 406
(±5 °C), the difference in mineralization degree should be 407
considered as a trend here. After SBF bath, the mineral content 408
increases for low rpDM1 concentrations (from ~3.5% to 409
~18%), whereas it does not change significantly at a high 410
content of rpDMP1 (from ~12.5% to ~15%). Since both 411
matrices exhibit spherulites on the surface after SBF bath 412
(Figure 2F and Figure S2C), it indicates that the main mineral 413
content is localized inside the matrix in the form of 414
nanoprecipitates. 415

In addition, the ζ-potential was investigated to help 416
understand the interactions between collagen, rpDMP1, and 417
the mineral. Before the SBF bath, the global surface charge 418
tends to be neutral in the presence of rpDMP1 whatever the 419
concentration of the protein (-0.79 (±0.66) mV and 0.22 420
(±0.78) mV) for low and high rpDMP1 concentrations, 421
respectively). Indeed, the Coll/SBF matrix exhibits a negative 422
ζ-potential (-4.3 (±2.5) mV).⁸ This result agrees with a partial 423
coating of collagen by the selected DMP1 domain, which 424
includes the two collagen binding sites²⁹ and the highly 425
conserved acidic peptide.³⁷ Indeed, it is reported that DMP1 426
binds to the N-telopeptide region in collagen.^{29,45} After 427
immersion in SBF, Coll/SBF(1rpDMP1)/SBF exhibits a more 428
negative ζ-potential (-9.77 (±0.48) mV), while it is slightly 429
unchanged for Coll/SBF(10rpDMP1)/SBF (-1.79 (±0.33) 430
mV). The ζ-potential of HA being negative between pH 5 and 431

432 8 (−5 to −37 mV respectively)⁶² is in agreement with the
433 TGA analysis.

434 To go deeper in the understanding of how the suprafibrillar
435 confinement affects the spreading of rpDMP1 over collagen,
436 the peptide was entrapped in a scaffold mimicking the fibrillar
437 arrangement of osteoid tissue (Figure 4A,B). As mentioned
438 above, this means that (i) the fibrils network is not as dense as
439 the previous mature bone model with larger interfibrillar spaces
440 which are above the micron size (macroporous gel) and (ii)
441 the collagen fibrils are not organized (no plywood geometry).
442 Note that spherulitic crystals form in the control matrix
443 without peptide (Coll/Osteoid/SBF/SBF) as shown by SEM
444 (Figure 4C) and TEM (Figure 4D), which is in agreement
445 with previous observations on a matrix with a collagen gradient
446 concentration.⁸

447 Interestingly, “dot-like” precipitates are also observed, but
448 they do not localize inside the fibrils (Figure 4A). Although
449 most of the investigations have focused on electrostatic
450 interactions to explain the intrafibrillar infiltration of both
451 mineral and proteins in collagen, this difference in apatite
452 crystals distribution in osteoid- and mature bone-like matrices
453 evidence that the suprafibrillar confinement provided by the
454 collagen assembly in bone tissue (i.e., cholesteric geometry)
455 plays a key role on this phenomenon. Thus, it may explain the
456 need of other proteins to infiltrate collagen in models lacking
457 biomimetic interfibrillar spaces (<1.7 nm).⁴⁵ The fact that
458 collagen here is continuously in contact with ions (even before
459 mineralization) as occurring in bone strengthens the need of a
460 balance between osmotic equilibrium and electroneutrality for
461 intrafibrillar mineralization.⁶³ In addition, the use of biomi-
462 metic collagen interfibrillar spaces (in both osteoid- and
463 mature-like matrices) contradicts that rpDMP1 favors the
464 templating of crystal growth.¹⁴ Conversely, according to (i)
465 TEM and SEM investigations where it is observed that the size
466 of spherulites consist of either “dot-like” aggregate or “mature”
467 apatite platelets with (Figures 2F, 3B, and 4A,B) or without
468 rpDMP1 (Figures 2G and 4C,D) respectively, (ii) the mineral
469 amount found by TGA, and (iii) the resulting surface charge
470 probed by ζ -potential, we confirm its role as a growth
471 inhibitor.³³ This effect may be related to the confinement
472 which promotes the protein folding by destabilizing the
473 unfolded state^{64,65} and thus further specific interactions
474 between rpDMP1 and apatite nuclei; the structural character-
475 istics of growing apatite being driven by the involvement of
476 ionic substitutions (specifically from carbonate ions).⁶⁶

477 **Toward Evidence of the Specific Activity of rpDMP1**
478 **under Confinement.** When polyAsp is supplemented to
479 collagen, it is difficult to observe a difference in mineral
480 spreading over the collagen matrix before immersion in SBF
481 (Coll/SBF(polyAsp), Figure 5A) as compared to rpDMP1.
482 Here also, a lower concentration of polyAsp (5.75 $\mu\text{g}/\text{mL}$) was
483 used than that in the literature (usually between 10 and 100
484 $\mu\text{g}/\text{mL}$) (i) to reach at least 2 magnitude order lower amounts
485 of negative charge and (ii) to reproduce a more “realistic”
486 collagen to NCPs ratio since such a high polyAsp
487 concentration can be used for one single fibril.³ Note that
488 our samples were not lyophilized before characterization, as it
489 is commonly performed with this polymer in the literature.⁶⁷
490 This is of importance since the resulting phase and degree of
491 mineralization are here related to a hydrated biomimetic
492 process. The difference between polyAsp and rpDMP1 in
493 apatite mineralization becomes clearer after the SBF bath
494 (Coll/SBF(polyAsp)/SBF, Figure 5B,C). TEM observations

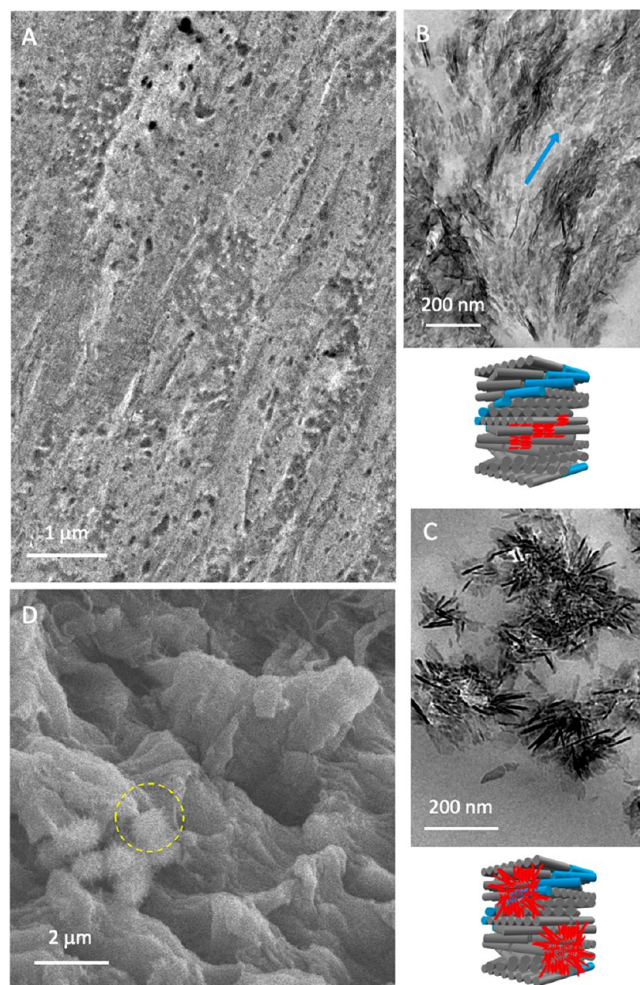


Figure 5. Calcium phosphate precipitation investigated by EM in the presence of polyAsp containing mature bone-like matrix. Observations by TEM of unstained ultrathin sections of matrices before and after SBF immersion: (A) Coll/SBF(polyAsp) and (B,C) Coll/SBF(polyAsp)/SBF, respectively. The corresponding schematic representation of the apatite crystal (red)/collagen fibril (gray) relationship in a 3D perspective is shown. The lack of staining in (A) indicates that there is an homogeneous spreading of SBF ions over the collagen. After immersion in SBF (B, C), HA platelets are observed in the matrix; they either (B) co-align with the long axis of the fibril (blue arrow) or (C) form spherulites as schematically presented, respectively, below the (B) and (C) TEM micrographs. (D) SEM micrograph of Coll/SBF(polyAsp)/SBF showing that spherulites (yellow dashed circle) are also observed inside the matrix.

495 reveal large domains where the mineral platelets co-align with
496 the collagen fibrils (Figure 5B), as seen in mature bone (Figure
497 1C) strengthening the affinity of polyAsp for gap regions in
498 line with previous conclusions in the literature.^{44,45,68}
499 However, in contrast to rpDMP1 results, nonbiomimetic
500 spherulitic crystals are also observed in the matrix (Figure
501 5C,D), strengthening that only small molecules such as
502 osteocalcin can penetrate alone the intrafibrillar space.⁶⁹

503 These observations evidence the specificity of biological
504 rpDMP1 versus synthetic polyAsp amino-acids sequences in
505 mediating apatite mineralization and also suggest that, in
506 addition to its role as growth inhibitor, it may prevent
507 homogeneous nucleation.³³ Finally, because the rpDMP1
508 sequence is not phosphorylated, this result tends to strengthen

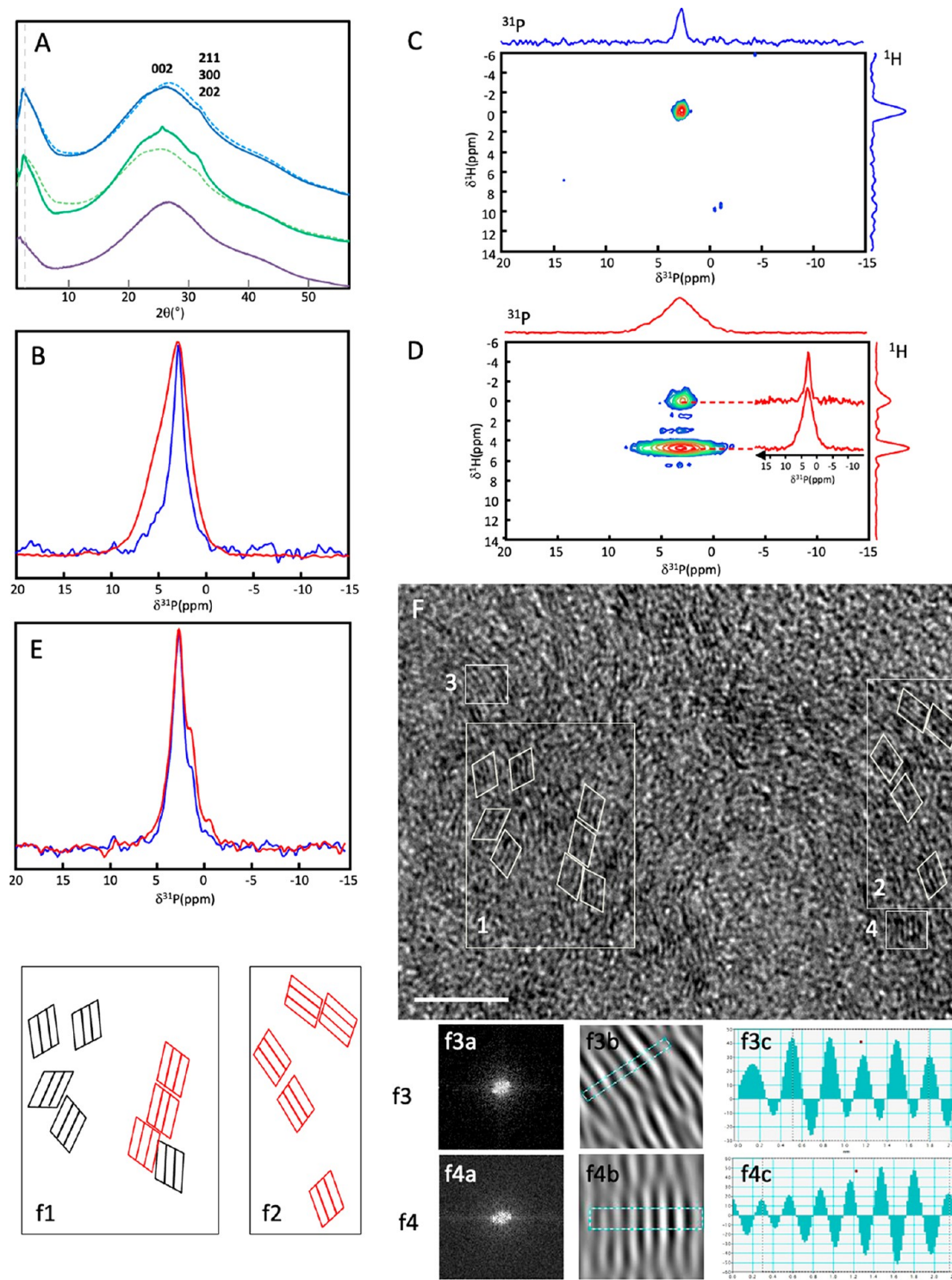


Figure 6. Characterization of the mineral phase. (A) 1D radial average of the WAXD patterns of matrices at 2.5 $\mu\text{g/mL}$, without (Coll/SBF(1rpDMP1), dashed green) and with (Coll/SBF(1rpDMP1)/SBF, plain green) immersion in SBF; at 25 $\mu\text{g/mL}$, without (Coll/SBF(10rpDMP1), dashed blue) and with (Coll/SBF(10rpDMP1)/SBF, full blue) immersion in SBF, and a collagen matrix as control (purple). The vertical gray dashed line points out the shift of the reflection which corresponds to the lateral distance between collagen molecules. (B) 1D ^{31}P CP MAS spectra (CT = 10 ms) of the hybrid matrices Coll/SBF(1rpDMP1) (blue) and Coll/SBF(10rpDMP1)/SBF (red). (C) 2D ^1H - ^{31}P HETCOR spectrum (CT = 10 ms) of Coll/SBF(1rpDMP1) with extracted ^{31}P slices at $\delta(^1\text{H}) = 0$ and 4.9 ppm. (D) 2D ^1H - ^{31}P HETCOR spectrum (CT = 10 ms) of Coll/SBF(10rpDMP1)/SBF. (E) 1D ^{31}P CP MAS spectra (CT = 10 ms) of the hybrid matrices Coll/SBF(10rpDMP1) (blue) and Coll/SBF(10rpDMP1)/SBF (red). (F) TEM micrograph of Coll/SBF(1rpDMP1)/SBF (scale bar = 5 nm) showing crystalline HA nanodomains. (f1) and (f2) are schematic representations of the selected areas 1 and 2 showing the orientation of the HA (210) planes; some crystalline nanodomains appear to align along the same direction (red). (f3) and (f4) FFT (f3a and f4a) and inverse FFT (f3b and f4b) performed on the selected areas 3 and 4, confirming the presence of crystalline nanodomains. The d spacings are measured using the inverse FFT profile along one direction (f3c and f4c).

509 that the organic phosphate concern may be attributed to
510 polyadenosine diphosphate ribose.⁷⁰

511 **Structural Features of rpDMP1-Mediated Mineral**
512 **under Confinement.** WAXD studies were performed to
513 better characterize the mineral phase and collagen network.
514 The 1D radial average of the WAXD patterns is shown in
515 Figure 6A. Without the SBF extra bath, the signal from
516 collagen fibrils is strong, revealing a low degree of
517 mineralization (dashed green and blue lines). The character-
518 istic HA diffraction peaks, that is, (002) and the merged (211),
519 (300), (202) reflections, are observed more clearly after the
520 SBF bath (full green and blue lines), especially for Coll/
521 SBF(1rpDMP1)/SBF, confirming an increase in mineral
522 content. In addition, the lateral distance between collagen
523 molecules decreases from 1.5 to 1.1 nm in the matrices loaded
524 with rpDMP1 (with or without SBF bath) in comparison to
525 the pure collagen matrix (full purple line) as observed by the
526 peak shift toward high angles (vertical gray dashed line). This
527 distance is shorter than that reported for a mineralized dense
528 collagen scaffold without protein,⁷ but agrees with densely
529 packed microfibrils⁷¹ and fibrillar collagen in a dry state.⁷²
530 Since the sample is studied in its native hydrated form, this
531 decrease in the intermolecular distance in addition to the
532 contrast observed in fibrils by TEM confirm that rpDMP1
533 localizes inside the gap zones. The peptide may colocalize with
534 the mineral as shown for polyelectrolytes.⁶³ Besides confine-
535 ment, SBF ions are needed for the protein to localize in
536 intrafibrillar spaces, especially calcium ions, considering the
537 model of a periodic assembly of DMP1 into a β -sheet template
538 with high calcium-binding capacity.¹⁴ Then the Ca-binding
539 protein can interact with a significant fraction of the π , that is,
540 possibly covalently bond to the collagen.⁷³ At a critical
541 concentration of Ca^{2+} , the apatite nucleation which strongly
542 depends on the degree of confinement⁷⁴ that occurs inside
543 collagen. However, since apatite forms without organic
544 additives under collagen confinement,⁸ it shows that
545 rpDMP1 increases the local ions concentration but does not
546 initiate the nucleation here.

547 To investigate the presence of calcium-phosphate minerals
548 described as transient phases in bone formation^{51,75,76} and
549 better understand the local phosphate environment in
550 collagen-containing rpDMP1, ³¹P ssNMR experiments were
551 performed (Figure 6B–E). The ³¹P CP MAS spectrum of
552 Coll/SBF(1rpDMP1) displays a single ³¹P resonance centered
553 at 2.8 ppm, typical of apatite (Figure 6B, blue). Interestingly,
554 the 2D ¹H–³¹P HETCOR spectrum of Coll/SBF(10rpDMP1)
555 does not show the correlation resonance characteristic from
556 HPO_4^{2-} (Figure 6C), excluding the presence of a hydrated
557 disordered surface layer.^{66,77} In addition, the line width of the
558 ³¹P resonance (LW = 1 ppm) is thinner than matrices prepared
559 without rpDMP1 (Coll/SBF, LW = 2 ppm) (Figure S4). Both
560 the thinner resonance and the absence/low amount of HPO_4^{2-}
561 surface species demonstrate that the mineral “dot-like”
562 precipitates of the matrices are highly crystalline and that
563 rpDMP1 drives the higher crystallinity of apatite. This result is
564 in agreement with previous works proposing that DMP1
565 impacts on the initial stages of apatite formation, providing a
566 structural local order.¹⁴ Interestingly, DMP1 is found more
567 abundant in the boundary between the dentin and enamel
568 where apatite is well crystallized.³⁰

569 Finally, it is interesting to note that such “dot-like crystals”
570 were found at the very early stages of normal *in vivo*
571 calcification.¹ After the SBF bath, the ³¹P LW in CP MAS

spectrum increases (Figure 6B, red), reaching the value found
572 for bone apatite (4.1 ppm).⁷⁸ The fact that the 2D ¹H–³¹P
573 HETCOR spectrum of Coll/SBF(1rpDMP1)/SBF (CT = 10
574 ms) (Figure 6D) displays the two typical resonances of bone
575 minerals, that is, $\delta(^{31}\text{P}) = 2.8$ and 3.2 ppm, shows that the
576 apatite spherulites observed at the surface of the matrices by
577 microscopy (Figure 2F) dominate the spectroscopic signature
578 here. 579

At a higher rpDMP1 content, 1D ³¹P CP-MAS spectra
580 (Figure 6E) display two shoulders (centered at 1.5 and –1
581 ppm) in addition to the apatite resonance at 2.8 ppm before
582 and after SBF. These resonances correspond to the HPO_4^{2-}
583 ions from the OCP phase. Noticeably, the presence of a
584 relatively low content of rpDMP1 leads to the physiological
585 single apatitic phase, whereas a high “nonphysiological”
586 concentration of protein stabilizes an additional CaP phase
587 like OCP. Such high concentrations are classically used in the
588 literature, questioning the role of DMP1 in stabilizing the ACP
589 phase.^{33,34} 590

HRTEM observations performed on ultrathin sections of
591 Coll/SBF(1rpDMP1)/SBF (Figure 6F and Figure S5) confirm
592 the highly crystalline nature of the “dot-like” precipitates.
593 Spaced thin layers that are mostly concentric, that is, onion-like
594 morphology that appears to be composed of crystalline
595 nanodomains (~1 nm) (some are depicted in areas 1 and
596 2), are observed. Schematic representations of two selected
597 areas (1 and 2) show the orientation of possible crystal planes
598 (f1 and f2). Some crystalline nanodomains appear to align
599 along the same direction (in red). Their morphology is difficult
600 to identify; they are presented here as parallelogram. In (f3)
601 and (f4), fast-fourier transform (FFT) (f3a and f4a) and
602 inverse FFT (f3b and f4b) performed on the (3 and 4) selected
603 areas confirm the presence of crystalline nanodomains. The
604 crystal planes were indexed by comparing the measured *d*
605 spacings using the FFT profile along one direction with
606 calculated values of HA (f3c and f4c). Two lattice spacings
607 were measured as 0.31 and 0.34 nm, corresponding to the
608 known (210) and (002) crystal planes of apatite (Figure 6F
609 and Figure S5, respectively). Among the 20 analyzed planes,
610 80% correspond to the (210) and the remaining 20% to the
611 (002). 612

The possible consequence of a crystalline apatite nano-
613 domain versus an ACP phase can prevent the formation of
614 pathological calcifications. Indeed, ACP as the first solid phase
615 of calcium phosphate formed in bone would delay apatite
616 formation, allowing the precipitation of other transient phases.
617 This hypothesis is in agreement with the fact that the
618 mineralization process of bone is described to occur rapidly
619 as soon as collagen molecules self-assemble into collagen fibrils
620 in the extracellular space.⁷⁹ In fact, according to our
621 knowledge, there is no report on bone pathologies related to
622 the presence of one of the transient apatite phases, although it
623 is extensively described in synthetic models lacking interfi-
624 brillar confinement *in vitro* as discussed above. 625

626 ■ CONCLUSION

The bone ECM is a dynamic (in terms of structure,
627 remodelling, and ECF) and complex environment where the
628 confinement effect occurs that is not only critical for the cells
629 behavior⁸⁰ but also for the proteins and even the solvent
630 (water).⁶⁴ Indeed, confinement provided by suprafibrillar
631 organization of collagen strongly impacts the activity of
632 mineralizing polymers. The results suggest the occurrence of 633

634 sequential cooperative effects during the early stages of bone
635 apatite precipitation through the formation of Ca-DMP1
636 complex, which then binds to collagen allowing the
637 concentration of apatite ion precursors in gap regions.
638 Formation of highly apatite nuclei and inhibition of both
639 homogeneous nucleation and crystal growth are consistent
640 with a common role of DMP1 controlling the physiological
641 (versus pathological) bone formation. Strikingly, these effects
642 are reached with the nonphosphorylated 57-KD amino acid
643 sequence under confinement. This versatile bone-like model
644 will be useful to provide insights into the role of other bone
645 components (e.g., citrate, proteoglycans, or even different
646 sequences related to NCPs kinetic of maturation (i.e., post-
647 translation modification)) during the successive events that
648 orchestrate mineralization.

649 ■ ASSOCIATED CONTENT

650 **SI** Supporting Information

651 The Supporting Information is available free of charge at
652 <https://pubs.acs.org/doi/10.1021/acs.biomac.1c00206>.

653 Composition of the solutions used to mineralize
654 collagen matrices, SEM and TEM images, EDX spectra,
655 and TGA curves (PDF)

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699 Notes

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