Matrix Macromolecules in Hard Tissues Control the Nucleation and Hierarchical Assembly of Hydroxyapatite

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Biogenic minerals found in teeth and bones are synthesized by precise cell-mediated mechanisms. They have superior mechanical properties due to their complex architecture. Control over biomineral properties can be accomplished by regulation of particle size, shape, crystal orientation, and polymorphic structure. In many organisms, biogenic minerals are assembled using a transient amorphous mineral phase. Here we report that organic components of bones and teeth, namely type I collagen and dentin matrix protein 1 (DMP1), are effective crystal modulators. They control nucleation of calcium phosphate polymorphs and the assembly of hierarchically ordered crystalline composite material. Both full-length recombinant DMP1 and post-translationally modified native DMP1 were able to nucleate hydroxyapatite (HAP) in the presence of type I collagen. However, the N-terminal domain of DMP1 (amino acid residues 1–334) inhibited HAP formation and stabilized the amorphous phase that was formed. During the nucleation and growth process, the initially formed metastable amorphous calcium phosphate phase transformed into thermodynamically stable crystalline hydroxyapatite in a precisely controlled manner. The organic matrix-mediated controlled transformation of amorphous calcium phosphate into crystalline HAP was confirmed by x-ray diffraction, selected area electron diffraction pattern, Raman spectroscopy, and elemental analysis. The mechanical properties of the protein-mediated HAP crystals were also determined as they reflect the material structure. Such understanding of biomolecule controls on biomineralization promises new insights into the controlled synthesis of crystalline structures.

Dentin consists of numerous noncollagenous proteins. Some of these macromolecules are generally highly acidic and often contain aspartic acid, glutamic acid, and serine. Such noncollagenous proteins are believed to provide the driving force required to reduce the activation energy of nucleation and formation of apatite.

Dentin matrix protein 1 (DMP1), a noncollagenous protein present in the mineral phase of bone and dentin in every species examined, has been studied as a model for proteins involved in the regulation of biomineralization (12–17). In addition to its potent calcium binding capacity, DMP1 binds with high affinity to fibrillar collagen (18). This complex organic matrix induces heterogeneous nucleation of calcium phosphate crystals and regulates crystal growth resulting in unique crystal morphologies (19). Deciphering the biological basis of biomineralization using tooth as a paradigm might unveil the mechanisms by which protein assemblies can determine the biomineral structure in vertebrates.

In this study, we discuss the hierarchical assembly of hydroxyapatite at the nanoscale level by demonstrating the formation of de novo amorphous calcium phosphate as an intermediate phase in the presence of an organic matrix, namely assembling properties and forms enclosed spaces within which the inorganic reinforcing phase grows. Various biochemical mechanisms and genetic regulations control the flux of organic components and maintain sufficient supersaturation of the mineral ions in the localized space. When the degree of supersaturation of calcium and phosphate ions is high, HAP, which is thermodynamically more stable, is formed via intermediate precursor polymorphs such as amorphous calcium phosphate (ACP), octacalcium phosphate, or dicalcium phosphate dihydrate (DCPD) (6, 7). It is well established that type I collagen matrix does not have the capacity to induce matrix-specific mineral formation from metastable calcium phosphate solutions that do not spontaneously precipitate but merely provide the organizational framework and spatial constraint for crystal deposition, whereas noncollagenous matrix macromolecules might be involved in the control of nucleation and growth of the mineral phase (8–11).

1193

2 The abbreviations used are: HAP, hydroxyapatite; DMP1, dentin matrix protein 1; GPa, gigapascal(s); XRD, x-ray diffraction; SAED, selected area electron diffraction; ACP, amorphous calcium phosphate; DCPD, dicalcium phosphate dihydrate; SEM, scanning electron microscopy; TEM, transmission electron microscopy; EDX, energy dispersive x-ray; Ca/P ratio, calcium to phosphate ratio; ICPS, inductively coupled plasma atomic emission spectroscopy; CDMP1, C-terminal DMP1; NDMP1, N-terminal DMP1; rDMP1, recombinant DMP1; ASTM, American Society for Testing and Materials Standards.

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Type I Collagen and DMP1 Nucleate Hydroxyapatite

type I collagen and DMP1. Subsequently the precipitates transformed into crystalline HAP structures with outstanding mechanical properties. We investigated the process of HAP nucleation using both recombinant and native DMP1 in the presence and absence of type I collagen, native and recombinant C-terminal DMP1 with and without type I collagen, and recombinant N-terminal DMP1 with and without type I collagen. We compared our in vitro data with the HAP crystal growth pattern in a rat incisor model.

Published reports demonstrate the use of two different crystal growth techniques, namely solution and gel techniques, for in vitro nucleation assays (20–22). Among these two methods, artificially formed gels and fibrous networks are excellent systems for studying crystallization because of the control they afford over local supersaturation and diffusion rates, and the physicochemical nature of this process more realistically mimics the mineralized tissue matrix environment (23, 24). In this study, sodium metasilicate gels were used as a crystallizing medium due to the following advantages. (a) Proteins are incorporated into the gel at room temperature, and hence they are not denatured and remain stable during the duration of the experiments. (b) The deposits are harvested with ease from the medium without contamination of sodium metasilicate. (c) The chemical reaction can be easily controlled. (d) The reaction medium is highly transparent so that the reaction can be easily observed.

MATERIALS AND METHODS

Expression and Purification of Type I Collagen

Type I collagen was isolated and purified as published earlier (25).

Expression and Purification of Recombinant DMP1

Recombinant DMP1 was expressed and purified as published earlier (26).

Cloning, Expression, and Purification of Recombinant C- and N-terminal DMP1

To identify the functional domain in DMP1 that was responsible for HAP nucleation, the coding sequence of rat DMP1 was subamplified into two parts by PCR with a set of primers flanking the required sequence region: 1) N term-S, G GAT CCC ATG AAG ACT GTT ATC CTC CTT ACC GAG TCT CAG GAA; 4) C-term-AS, GGC CGC TTC CTG GGA TCC CTC ACC AGC; 2) N term-AS, GC GGC CGC GTA GCC ATC TTG GCA ATC ATT.

Expression and Purification of Recombinant C- and N-terminal DMP1

Recombinant DMP1 was expressed and purified as published earlier (26).

Retention of the supernatant solution

Initial concentration in the medium Stability of the medium Gelation time Concentration of the calcium ions in the gel medium

| g/ml | Unstable | 4 days | 3.41 ± 0.20 | 4.1 ± 0.11 |
| 1.01 | 70% stable, transparent medium | 2 days | 2.61 ± 0.09 | 3.24 ± 0.06 |
| 1.03 | 100% stable, transparent medium | 12–14 h | 2.06 ± 0.10 | 2.04 ± 0.04 |
| 1.04 | 100% highly stable, opaque | 6–7 h | 1.62 ± 0.12 | 1.64 ± 0.09 |
| 1.05 | 100% highly stable, highly opaque | 2 h | 1.62 ± 0.12 | 1.64 ± 0.09 |

Concentration of calcium ions diffused into the gel medium

Initial concentration in the medium | Concentration of calcium ions diffused into the gel medium

| Initial concentration in the medium | 10 days | 20 days | 30 days |
| g/ml | mg/ml | mg/ml | mg/ml |
| 0 | 0 | 0 | 0 |
| 10 | 0.24 ± 0.06 | 0.36 ± 0.03 | 0.51 ± 0.04 |
| 20 | 0.51 ± 0.04 | 0.81 ± 0.02 | 0.89 ± 0.02 |
| 40 | 0.86 ± 0.03 | 1.21 ± 0.03 | 1.34 ± 0.03 |
| 60 | 1.31 ± 0.05 | 1.91 ± 0.07 | 2.10 ± 0.04 |
| 80 | 1.87 ± 0.07 | 2.32 ± 0.02 | 2.54 ± 0.03 |
| 100 | 2.61 ± 0.12 | 3.27 ± 0.05 | 3.79 ± 0.07 |
| 250 | 4.89 ± 0.08 | 6.0 ± 0.04 | 6.47 ± 0.02 |
| 500 | 10.21 ± 0.24 | 14.8 ± 0.08 | 15.2 ± 0.20 |

Concentration of diffused calcium ions into the sodium metasilicate (specific gravity, 1.03 g/ml) gel medium

| Specific gravity of the medium | Stability of the gel medium | Gelation time | Concentration of the calcium ions in the crystallizing medium |
| g/ml | | | Day 0 | Day 7 | Day 10 |
| --- | --- | --- | --- | --- | --- |
| 1.01 | Unstable | 4 days | 2.61 ± 0.09 | 2.61 ± 0.09 |
| 1.02 | 70% stable, transparent medium | 2 days | 2.61 ± 0.09 | 2.61 ± 0.09 |
| 1.03 | 100% stable, transparent medium | 12–14 h | 2.61 ± 0.09 | 2.61 ± 0.09 |
| 1.04 | 100% highly stable, opaque | 6–7 h | 2.61 ± 0.09 | 2.61 ± 0.09 |
| 1.05 | 100% highly stable, highly opaque | 2 h | 2.61 ± 0.09 | 2.61 ± 0.09 |

In vitro mineralization experimental conditions

Experiment serial no. | Reactants | Concentration of diffused calcium ions in the crystallizing medium |
| --- | --- | --- |
| | | Day 0 | Day 7 | Day 10 |
| 1 | 1.61 mM KH₂PO₄ | 0 | 2.46 ± 0.06 | 2.61 ± 0.09 |
| 2 | 1.61 mM KH₂PO₄ + 5 μg/ml type I collagen | 0 | 2.46 ± 0.06 | 2.61 ± 0.09 |
| 3 | 1.61 mM KH₂PO₄ + 25 μg/ml rDMP1 | 0 | 2.46 ± 0.06 | 2.61 ± 0.09 |
| 4 | 1.61 mM KH₂PO₄ + 25 μg/ml rDMP1 + 5 μg/ml type I collagen | 0 | 2.46 ± 0.06 | 2.61 ± 0.09 |
| 5 | 1.61 mM KH₂PO₄ + 25 μg/ml native DMP1 | 0 | 2.46 ± 0.06 | 2.61 ± 0.09 |
| 6 | 1.61 mM KH₂PO₄ + 25 μg/ml native DMP1 + 5 μg/ml type I collagen | 0 | 2.46 ± 0.06 | 2.61 ± 0.09 |
| 7 | 1.61 mM KH₂PO₄ + 25 μg/ml rCDMP1 | 0 | 2.46 ± 0.06 | 2.61 ± 0.09 |
| 8 | 1.61 mM KH₂PO₄ + 25 μg/ml rCDMP1 + 5 μg/ml type I collagen | 0 | 2.46 ± 0.06 | 2.61 ± 0.09 |
| 9 | 1.61 mM KH₂PO₄ + 25 μg/ml native CDMP1 | 0 | 2.46 ± 0.06 | 2.61 ± 0.09 |
| 10 | 1.61 mM KH₂PO₄ + 25 μg/ml native CDMP1 + 5 μg/ml type I collagen | 0 | 2.46 ± 0.06 | 2.61 ± 0.09 |
| 11 | 1.61 mM KH₂PO₄ + 25 μg/ml rNDMP1 | 0 | 2.46 ± 0.06 | 2.61 ± 0.09 |
| 12 | 1.61 mM KH₂PO₄ + 25 μg/ml rNDMP1 + 5 μg/ml type I collagen | 0 | 2.46 ± 0.06 | 2.61 ± 0.09 |
Type I Collagen and DMP1 Nucleate Hydroxyapatite

**TABLE 4**

Elemental analysis by EDX and ICPAES

| Experiment serial number | Calculated Ca/P ratio |  |
|--------------------------|-----------------------|--|
|                          | EDX analysis | ICPAES analysis | Day 7 | Day 60 | Day 7 | Day 60 |
| 1                        | 1.48 ± 0.03 | 0.98 ± 0.05 | 1.52 | 1.02 |
| 2                        | 1.48 ± 0.03 | 1.01 ± 0.03 | 1.51 | 1.05 |
| 3                        | 1.48 ± 0.03 | 1.62 ± 0.01 | 1.47 | 1.62 |
| 4                        | 1.49 ± 0.02 | 1.65 ± 0.02 | 1.50 | 1.64 |
| 5                        | 1.46 ± 0.05 | 1.61 ± 0.02 | 1.48 | 1.63 |
| 6                        | 1.46 ± 0.05 | 1.67 ± 0.02 | 1.47 | 1.67 |
| 7                        | 1.51 ± 0.03 | 1.64 ± 0.03 | 1.48 | 1.63 |
| 8                        | 1.51 ± 0.03 | 1.66 ± 0.02 | 1.47 | 1.66 |
| 9                        | 1.51 ± 0.03 | 1.63 ± 0.02 | 1.49 | 1.64 |
| 10                       | 1.51 ± 0.03 | 1.69 ± 0.04 | 1.51 | 1.71 |
| 11                       | 1.47 ± 0.02 | 1.49 ± 0.02 | 1.53 | 1.53 |
| 12                       | 1.47 ± 0.02 | 1.47 ± 0.01 | 1.52 | 1.49 |

**FIGURE 1.** SEM, electron diffraction patterns, and EDX patterns of calcium phosphate deposits. a, initial amorphous deposits; inset, diffused electron diffraction pattern. b, SEM of spherulitic DCPD crystal grown in the absence of proteins. c, microstructure of DCPD crystals mediated by type I collagen only. d, EDX spectra confirms the presence of calcium and phosphate ions: amorphous calcium phosphate (blue), DCPD without protein (pink), DCPD formed in the presence of type I collagen (red). e, SEM of platy DCPD crystals mediated by type I collagen only. f, powder x-ray diffraction pattern of amorphous calcium phosphate. g, EDX of DCPD formed without proteins. h, EDX of DCPD crystals grown in the presence of type I collagen. a.u., absorbance units.

**TABLE 3**

EDX analysis of DCPD formed without proteins. a, absorption units.

| Experiment serial number | EDX analysis | Day 7 | Day 60 |
|--------------------------|--------------|-------|--------|
| 1                        | 1.48 ± 0.03  | 0.98 ± 0.05 | 1.52 | 1.02 |
| 2                        | 1.48 ± 0.03  | 1.01 ± 0.03 | 1.51 | 1.05 |
| 3                        | 1.48 ± 0.03  | 1.62 ± 0.01 | 1.47 | 1.62 |
| 4                        | 1.49 ± 0.02  | 1.65 ± 0.02 | 1.50 | 1.64 |
| 5                        | 1.46 ± 0.05  | 1.61 ± 0.02 | 1.48 | 1.63 |
| 6                        | 1.46 ± 0.05  | 1.67 ± 0.02 | 1.47 | 1.67 |
| 7                        | 1.51 ± 0.03  | 1.64 ± 0.03 | 1.48 | 1.63 |
| 8                        | 1.51 ± 0.03  | 1.66 ± 0.02 | 1.47 | 1.66 |
| 9                        | 1.51 ± 0.03  | 1.63 ± 0.02 | 1.49 | 1.64 |
| 10                       | 1.51 ± 0.03  | 1.69 ± 0.04 | 1.51 | 1.71 |
| 11                       | 1.47 ± 0.02  | 1.49 ± 0.02 | 1.53 | 1.53 |
| 12                       | 1.47 ± 0.02  | 1.47 ± 0.01 | 1.52 | 1.49 |

*richia coli* BL21-DE3 cells (Invitrogen). The glutathione S-transferase fusion protein induced and purified by the standard procedure was cleaved by thrombin at 4 °C. The corresponding peptides were termed NDMP1 (residues 1–334) and CDMP1 (residues 334–489).

**Native DMP1 and CDMP1**

Native DMP1 and CDMP1 used in *in vitro* nucleation experiments were isolated from rat bone and were kind gifts from Dr. Chunlin Qin (27).

**In Vitro Crystallization**

Calcium phosphates were crystallized by the slow and controlled chemical reaction between the calcium and phosphate ions (with or without organic matrix) in a semisolid medium at physiological pH and temperature. The semisolid crystallizing medium was prepared by using 10 ml of sodium metasilicate solution of specific gravity 1.06 g/ml, 2.7 ml of 60 mM KH2PO4, 1 ml of HEPES buffer, pH 7.4, and 87.3 ml of deionized water. The final composition of the medium was 1.03 g/ml sodium metasilicate, 1.61 mM KH2PO4, 10 mM HEPES, pH 7.4. Protein solutions were prepared in 10 mM HEPES pH 7.4 buffer and then mixed with the sodium metasilicate solution (SMS). The SMS solution was then allowed to polymerize incorporating the protein in the crystallizing medium. After gelation, the second reactant (100 mM Ca2+ in 10 mM HEPES buffer, pH 7.4) was added over the crystallizing medium. Due to the diffusion process, the calcium ion concentration reaches 2.61 ± 0.09 mM at the end of day 10; therefore, the top solution was replaced with 2 ml of 50 mM HEPES buffer, pH 7.4. The concentration of calcium ions that diffused into the crystallizing medium with respect to time and also the influence of specific gravity on the stability of the gel are presented in Table 1 and Table 2. Twelve sets of experiments were conducted to elucidate the influence of type I collagen and either recombinant or native DMP1, native or recombinant CDMP1, or recombinant NDMP1 on the nucleation, phase transformation, morphology, and microstructure of the precipitated calcium phosphate minerals. The details of the experimental conditions are presented in Table 3.

**Sample Preparation for Analysis**

At different time points (days 7, 14, 21, 28, 35, 42, 48, and 60) the calcium phosphate deposits were harvested and separated from the silica medium. The intact mineral deposits were directly used for scanning electron microscopy (SEM) studies. For all other studies, the material was ground into fine crystallites and used for further analysis.

**Characterization of the Mineral Deposits**

The following methods were used to characterize the mineral deposits.

*Scanning Electron Microscopy*—The morphology and microstructure of the calcium phosphates (with or without the organic matrix) were examined at different time intervals using a scanning electron microscope (Hitachi 3000). The samples were mounted on an aluminum stub using double sticky tape and then coated with gold (JEOL JFC-1200 fine coater) by a sputtering technique at a vacuum of 10⁻⁸ torr.
**Type I Collagen and DMP1 Nucleate Hydroxyapatite**

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**X-ray Diffraction (XRD) Studies**—The XRD patterns of the powdered samples obtained from experiments 1–4 at different time intervals were recorded from 10° 2θ to 42° 2θ with a Rigaku D Max 2200 diffractometer operated at 40 kV and 40 mA, producing CuKα radiation with wavelength λ = 1.5418 Å, and at a scan speed of 0.02°/min. The relative intensities were determined as diffraction line heights relative to the most intense line normalized to the intensity of the (100) plane with the Materials Data Inc. JADE 6.1 XRD patterns processing software (MDI JADE 6.1). For crystallite size and crystal-lineity determinations, the spectra were recorded at an interval 25° ≤ 2θ ≤ 27° to obtain the single diffraction peak along the (002) plane. The scan speed was 0.02°/min, and the step size was 0.01°.

**Raman Spectroscopic Analysis**—Raman spectra were recorded with a Renishaw 2000 spectrometer in the range from 1500 to 500 cm⁻¹ using 514.5 nm wavelength excitation from an air-cooled argon ion laser with a power of 30 milliwatts. A holographic notch filter was used to filter out the Rayleigh radiation. The Raman radiation was dispersed using an 1800 groove/mm grating and detected by a Peltier cooled charge-coupled device array.

**Transmission Electron Microscopy (TEM) Analysis**—JEOL 3010 transmission electron microscope was used to analyze the calcium phosphates mediated by (a) native full-length DMP1, (b) native full-length DMP1 + type I collagen, (c) recombinant and native CDMP1, (d) recombinant CDMP1 + type I collagen, (e) native CDMP1 + type I collagen, (f) recombinant NDMP1, and (g) recombinant NDMP1 + type I collagen. For TEM studies, the calcium phosphate precipitates were separated by ultrasonic vibration in an ethanol medium and then placed on carbon Formvar-coated nickel grids. TEM was also performed on rat incisor, and the morphology and the selected area electron diffraction (SAED) pattern were determined for each of the regions marked A, B, and C.

**Elemental Analysis**

The elements present in the mineral deposits (experimental setups 1–4) were determined using an energy dispersive x-ray
(EDX) apparatus attached to a scanning electron microscope. EDX (Vista model, Thermo Noran) coupled with TEM was used for samples from experimental setups 5–12 and also for rat teeth sections A, B, and C. The calcium to phosphate (Ca/P) ratio was calculated from the intensity of the peaks present in the EDX pattern, and these values were compared with the values obtained from ICPAES analysis (Varian inductively coupled plasma atomic emission spectrometer). For ICPAES analysis, 250 mg of calcium phosphates were dissolved in 10 ml of dilute hydrochloric acid (0.1%).

**Determination of the Crystallinity and Crystallite Size of HAP**

The crystallinity \( (1/\beta) \) and crystallite size \( (L_c) \) of the spherulitic HAP crystals at the end of the maximum growth period mediated by DMP1 was calculated using Scherrer’s equation,

\[
L_c = \frac{k\lambda}{\beta \cos \theta},
\]

(Eq. 1)

where \( k \) is the shape coefficient (approximately 0.9 – 1.0), \( \lambda \) is the wave length, \( B \) is the full width at half-maximum of each plane, and \( \theta \) is the diffraction angle. The corrected value of angular width \( (\beta) \) was calculated from \( \beta = (B^2 - b^2)^{1/2} \) where “b” is the instrumental broadening \( (b = 0.087^\circ) \) calculated from a well crystallized apatite (28).

**Mechanical Properties**

For mechanical studies, the protein mediated (rDMP1/rDMP1 + type I collagen) hydroxyapatite crystals were ground into fine crystallites, and then the “dry powder press method” was used to prepare a thin HAP pellet of 2-mm thickness. Young’s modulus and hardness of the HAP crystalline pellets were measured using the nanoindenter (TriboIndenter, Hysitron Inc., Minneapolis, MN). A loading/unloading rate of 0.0002 newton and an applied load of 0.001 newton were used. The nanoindenter software (TriboScan, version 6.0.0.31, Hysitron Inc.) calculates the hardness by dividing the load by the surface area. The elastic modulus of the composite biomimeral was calculated from the following equation,

\[
1/E_r = (1 - n^2)/E + (1 - n_o^2)/E_o
\]

(Eq. 2)

where \( E_r \) is the reduced modulus from the nanoindenter, \( E \) is the modulus of the Berkovich diamond indenter (1141 GPa), \( E_o \) is the modulus of the HAP material, \( n \) is Poisson’s ratio for the indenter (0.07), and \( n_o \) is Poisson’s ratio for the composite (0.28).

The reduced modulus \( (E_r) \) was determined from the corrected contact stiffness, \( S_r \). The contact areas for each indentation \( (A) \) were obtained from a tip shape calibration procedure.

\[
E = ((\pi)^{1/2}/2A^{1/2})S_r
\]

(Eq. 3)

\( S_r \) is the contact stiffness obtained from the derivative of the unloading curve evaluated at peak force.

**RESULTS**

**Characterization of the Calcium Phosphate Deposits Obtained Only in the Presence of Type I Collagen—Micrometer-sized calcium phosphate crystals having a platy morphology were precipitated in the presence of type I collagen (Fig. 1a). The number of nucleation sites was found to increase gradually after day 7 predominantly in the area where amorphous calcium phosphate was first deposited (Fig. 1a). At the end of 20th day the amorphous structures were completely replaced by platy calcium phosphate crystals, confirmed as DCPD by XRD (Fig. 1h), as the major reflection peak at \( 2\theta = 11.9^\circ \) corresponds to (010) plane and indicates growth along the b axis. Elemental analysis confirmed the composition (Ca/P, 1.01 ± 0.03) to be DCPD (Fig. 1e). These results demonstrate that although collagen is the major organic component of the mineralized matrix of bone and dentin, it could not initiate nucleation of HAP. These data correlate well with our earlier published data (30).**

**Characterization of Spherulitic Apatite Formed Only in the Presence of rDMP1—in the presence of rDMP1, precipitates of amorphous calcium phosphate was observed after 7 days of growth, and the x-ray diffraction analysis demonstrated a broad**

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**Table 5**

Calculated crystallite size of the spherulitic and platy hydroxyapatite crystals grown in the presence of DMP1 and collagen + DMP1 (along c axis direction (002))

| Growth time | Crystallite size \((L_c)\) (002) plane |
|-------------|-----------------------------------|
|             | DMP1                               | DMP1 + collagen                    |
| days        | \(A^\circ\)                        | \(A^\circ\)                       |
| 0           | 0                                  | 0                                |
| 7           | 0                                  | 0                                |
| 14          | 3.69                               | 4.08                             |
| 21          | 4.048                              | 4.92                             |
| 28          | 4.57                              | 5.31                             |
| 35          | 4.93                              | 5.95                             |
| 42          | 5.45                              | 8.11                             |
| 49          | 6.27                              | 8.11                             |
| 60          | 6.27                              | 8.11                             |

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3 ASTM powder diffraction file compiled by Joint Committee on Powder Diffraction Studies, 1983 [00-011-0293].
Type I Collagen and DMP1 Nucleate Hydroxyapatite
peak in the region between $2\theta = 15^\circ$ and $2\theta = 32^\circ$ confirming that the harvested material was amorphous in nature (Fig. 2j). Furthermore, the diffused ring pattern from electron diffraction (Fig. 2a, top left), a predominant band at 950 cm$^{-1}$ ($v_1(PO_4^{3-})$) in the Raman spectra (Fig. 2k), and the Ca/P ratio of 1.48 ± 0.03 additively confirmed the amorphous nature of the polymorph (Fig. 2i). After 14 days, the precipitate gradually transformed into spherulites of size 90 ± 6.1 μm (Fig. 2, a–f). SEM analysis on the microstructure of the spherulites revealed the presence of a radially arranged aggregation of single fibrous crystals of ~70 nm in size (Fig. 2, g and h). With further crystal maturation, XRD analysis demonstrated an increase in the intensity of the 20 peak at 25.6° and 31.6° indicative of growth along the (002) plane and the (211) plane, respectively. The $d$ values calculated from the XRD pattern at day 60 (Fig. 2j) and electron diffraction pattern (Fig. 2g, top left) confirmed that the spherulitic material was HAP and were in good agreement with the standard ASTM values. An increase in the intensity of the 960 cm$^{-1}$ band in the Raman spectra with crystal maturation confirmed the transformation from poorly crystalline to highly crystalline HAP (Fig. 2k). The crystalline lattice of spherulitic HAP was confirmed by EDX (Fig. 2i), and the Ca/P ratio calculated from ICPAES and EDX was found to be nearly equal to the theoretical Ca/P ratio (1.67) for HAP (Table 4). Fig. 4a shows the calculated crystallinity $1/\beta$, and the corresponding calculated crystallite size along the c axis of spherulitic HAP (at the end of 60 days) are presented in Table 5. After an initial critical size attained within 7 days, the values increased steadily until a steady state was reached with crystal maturation attained after the 49th day. Further maturation did not yield any apparent increase in crystallinity or increase in crystallite size along the c axis.

Effect of rDMP1 and Type I Collagen on Apatite Formation—Combination of type I collagen and rDMP1 had the most profound effect on HAP nucleation and growth. SEM analysis in Fig. 3a demonstrated that the calcium phosphate deposits at the end of 7 days of growth had a unique coiled morphology, and such spherical shapes are known to minimize the area of interfacial tension with the surrounding solution. The spontaneous formation of ACP in the presence of the organic matrix is a kinetically driven process. Symmetric stretch of the phosphate at 950 cm$^{-1}$ in the Raman spectra (data not shown) confirmed that these unique structures were amorphous in nature. Furthermore the calculated Ca/P ratio was 1.49 ± 0.02, consistent with the empirical formula Ca$_3$(PO$_4$)$_2$ (Fig. 3i). By the end of 9 days of growth, several needle-shaped crystals emerged from the amorphous phase in a controlled manner (Fig. 3b). The density of the needle-shaped nanocrystallites increased after 28 days and was accompanied by a change in the width of the crystals (Fig. 3, c–f). A predominant band at 960 cm$^{-1}$ in the Raman spectra was very intense after 28 days confirming the presence of HAP (Fig. 3j). This vibration is associated with the symmetric $v_1$(PO$_4$) stretching mode of the free tetrahedral phosphate ion (Fig. 3j). The shift of the phosphate band from 950 to 960 cm$^{-1}$ confirmed the conversion of ACP to highly crystalline HAP. An increase in the intensity of the 960 cm$^{-1}$ band with crystal maturation confirmed the highly crystalline nature of mature HAP. At the end of 60 days, the plate-shaped morphology of the single crystals (Fig. 3g) resembled the HAP crystals in a continuously erupting rat incisor (Fig. 3h).
Type I Collagen and DMP1 Nucleate Hydroxyapatite

SAED pattern (Fig. 5d), EDX (data not shown), ICPAES (Table 4), and Raman spectrum (Fig. 5h) confirmed that the calcium phosphate harvested at the end of the 7th day had characteristic features of the amorphous phase. The morphology and microstructure of the calcium phosphate particles harvested at the end of 49th day (Fig. 5, b, c, and e) demonstrate formation of spherulites similar to the crystals obtained with rDMP1; however, the calculated Ca/P ratio was less (1.61 ± 0.02). In the presence of type I collagen, the HAP crystals exhibited needle-shaped morphology (Fig. 5, f and g) with a calculated Ca/P ratio of 1.67 ± 0.02. Their d values (Fig. 5, e and g, top left) and the data from the Raman spectrum (Fig. 5, i and j) confirmed that the deposits were HAP.

Characterization of the Mineral Deposits Formed in the Presence of rCDMP1, Native CDMP1, and rNDMP1 in the Presence and Absence of Type I Collagen—Published reports (27) demonstrate that DMP1 can be proteolytically cleaved into an N-terminal and a C-terminal fragment. To determine the HAP nucleating capacity of the two polypeptides; the NDMP1 and CDMP1 fragments were expressed recombinantly and used for in vitro nucleation assays. Results obtained with the rCDMP1 mimicked exactly the data obtained from full-length recombinant and native DMP1 (Fig. 6). However, in the presence of type I collagen + rCDMP1 distinct needle-shaped crystals (Fig. 6, e and f) were obtained, and these were confirmed as HAP by the same methods described above (data not shown). The calculated Ca/P ratio was found to be the same as the theoretical ratio for HAP (Table 4).

In vitro nucleation experiments were also performed with native CDMP1 in the presence and absence of type I collagen. Spherulites of 30 ± 2 μm were obtained in the absence of collagen (Fig. 6g). These spherulites were composed of uniformly and radially arranged needle-shaped crystals of size 40 ± 5 nm, and the crystals (Fig. 6h) were identified as HAP by SAED pattern (Fig. 6i) and also by Raman spectroscopy (data not shown). In the presence of collagen, bundles of needle-shaped HAP crystals were obtained with high crystallinity (Fig. 6, j and k). HAP polymorph was confirmed by the calculated Ca/P ratio (1.66 ± 0.02) (Table 4) and also from the characteristic peak for PO4 3− (960 cm−1) in the Raman spectrum. In all the above experiments (from experimental setups 7–10), the material harvested at the end of 7th day was amorphous and possessed an irregular morphology (Fig. 6a). The amorphous nature was further confirmed by the diffused ring pattern (SAED) (Fig. 6b, top left) and Raman spectrum (data not shown).

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Interestingly in vitro nucleation experiments conducted with type I collagen and rNDMP1 deposited precipitates that were amorphous, and they failed to transform into HAP even after 49 days of growth (Fig. 6, l and m). This clearly demonstrates that the N-terminal DMP1 polypeptide had the capacity to stabilize the amorphous calcium phosphate phase. The EDX spectrum (not shown) and ICPAES (Table 4) confirmed that the deposits mediated by NDMP1 at the end of the 7th and 49th days were amorphous (1.47 ± 0.02 to 1.48 ± 0.03). This ratio remained the same in the presence and absence of type I collagen. Further characterization by Raman spectroscopy confirmed that the polymorphs were ACP (data not shown).

Characterization of the Biomineralization Events in a Developing Rat Incisor—A developing rat incisor is a good model to study the morphology and composition of the calcium phosphate crystals at all stages of the dentin assembly process. In the results from the SEM, the region denoted “A” in Fig. 7A represents an area enriched with collagenous fibers. The region denoted “B” in Fig. 7B depicts an area where mineralization had just begun, and this was confirmed by the diffused SAED pattern as a “poorly crystalline” region corresponding to the start of the mineralization phase. Region “C” in Fig. 7C represents a highly mineralized area where the calcium phosphate deposits had a platelike morphology (needle-like when tilted on its edge). The mineral in this region was identified as HAP from the SAED pattern. EDX values obtained from these three sites (Fig. 7D) showed a change in the Ca/P ratio from 1.47 to 1.61 indicating a transformation from an amorphous to a crystalline phase. These results are in good agreement with our in vitro data.

Crystallinity of HAP Mediated by Native and Recombinant DMP1—The crystallinity of the deposits mediated by the native proteins were calculated from the XRD pattern (along (002) growth plane) and compared with the recombinant proteins (Fig. 8). The crystallinity of the HAP mediated by native DMP1 was less than that of the rDMP1, whereas the crystallinity (1/β) of the HAP mediated by native CDMP1 was more than that mediated by rCDMP1. These variations may be due to the differences in the amino acid residues and the presence of phosphorylated serines in native DMP1. In the presence of type I
collagen the crystallinity of HAP was much higher than that in the presence of DMP1 by itself. This could be attributed to the growth of HAP within the constrained space defined by type I collagen.

**Elastic Modulus and Hardness of Apatite Mediated by rDMP1 and rDMP1 + Type I Collagen**—As the mechanical properties reflect the material structure, therefore in this study biomechanical properties were determined for HAP crystals grown in the presence of proteins. The elastic moduli of HAP crystals mediated by rDMP1 and DMP1 in combination with type I collagen were studied using the nanoindentation technique. Typical force/displacement profiles from which the elastic modulus $E$ and hardness $H$ were derived for both samples are shown in Fig. 9, a and b. The elastic modulus of the rDMP1-mediated crystalline HAP was found to be $6.58 \pm 1.02$ GPa, whereas the elastic modulus of platy HAP crystals grown in the presence of type I collagen and rDMP1 was found to be $18.08 \pm 1.28$ GPa. These values are comparable with the elastic moduli reported (32–35) for various types of bone and dentine (Fig. 9c). Overall these results clearly demonstrate that the Young’s modulus for HAP obtained in the presence of type I collagen + rDMP1 was found to be ~5.8% more than the reported elastic modulus of dentin (33) and 0.9% more than that of bone indicating the high crystallinity of the material formed in the presence of the organic matrix.

The calculated hardness for the DMP1-mediated apatite was found to be $0.09076 \pm 0.03$ GPa, whereas for DMP1 + type I collagen it was $0.5212 \pm 0.13$ GPa. This value was on par with the reported value of $0.49 – 0.52$ GPa for the hardness of dentin near the dentino-enamel junction (36). Increase in hardness and elastic modulus with DMP1- and collagen-mediated HAP crystals demonstrates an increase in the mineral content.

**DISCUSSION**

A commonly used strategy in biomineralization is the presence of an extracellular organic matrix. In this study we demonstrated that biologically derived materials that form hard tissues such as bone and teeth can be synthesized in the presence of macromolecules such as type I collagen and dentin matrix protein 1 with extraordinary biomechanical properties. Type I collagen...
collagen defines the space in which the crystal grows, and this constrained space might be necessary for defining the crystal size and morphology. Acidic proteins like DMP1 are important constituents of the organic matrices of mineralized tissues and are involved in the nuleation, transformation, and growth of HAP. Thus, acidic macromolecules can initiate and stabilize non-equilibrium crystal polymorphs by their macromolecular confirmation and the microenvironment surrounding the nucleating phase.

A remarkable feature noted from this study is that both type I collagen and DMP1 were required for the synthesis of needle-shaped HAP crystals with high crystallinity. Type I collagen by itself does not function to nucleate HAP; however, we have demonstrated earlier that cooperative interaction between DMP1 and collagen matrix could be an essential step in the biomineralization process in matrix-mediated mineralization (19). To delineate the domain(s) in DMP1 that was responsible for HAP nucleation, two recombinant polypeptides comprising residues 1–334, designated as NDMP1, and 334–489, designated as CDMP1, were expressed and used in in vitro assays. Results from the study demonstrated that the CDMP1 polypeptide was responsible for the nucleation activity. Post-translationally modified native CDMP1 fragment comprising residues 170–489 also promoted HAP nucleation and growth. It thus appears that the charged residues present at the C-terminal end of DMP1 are spatially organized in a form that specifically complements the crystallography of the calcium phosphate nuclei. A model presented in Fig 10 (obtained by using "MyHits" software developed by the Swiss Institute of Bioinformatics) depicts the N terminus of DMP1 as an aspartic acid-rich domain and the C terminus as a glutamic acid- and serine-rich domain (37). Thus, the glutamic acid and serine residues might be directly responsible for HAP nucleation. After the formation of the initial amorphous phase, structural rearrangement of the dispersed ionic cluster then takes place into more ordered structures dictated by matrix interactions. Similarly the nucleating activity of bone sialoprotein is believed to reside primarily in two polyglutamic acid domains found in the N-terminal half of the molecule (38). Chondroitin sulfate has also been shown to improve the interfacial structure match between HAP crystallites and the substrates during the nucleation process (39). Our investigations also strongly suggest that the mineralized matrix might be assembled through an amorphous precursor.
phase. Interestingly in the present study we demonstrated that the amino acid sequences at the N terminus of DMP1 can stabilize the amorphous phase. The high charge density created by the aspartic acid residues at the N terminus of DMP1 can bind calcium ions very strongly favoring formation and stabilization of the amorphous nuclei. The stabilization of amorphous precursors during the growth phase is important in biomineralization because transient amorphous minerals play an important role in many organisms. It is well known that amorphous materials can be molded into various shapes; moreover amorphous calcium phosphate is known to have a curvilinear appearance rather than a faceted, angular shape of crystalline calcium phosphates. A growing body of evidence has shown that the shape of the biominerals is often controlled through molding of solid or gelated amorphous precursors (29). Transition of amorphous phase to crystalline phase is carried out in a controlled manner by organisms. Recent studies have demonstrated that sea urchin spine regeneration proceeds via the initial deposition of amorphous calcium carbonate (29).

It is also known that amorphous calcium phosphates may play a significant role in pathological calcifications and in diseases like osteoarthritis. Calcium phosphate in milk is stored as ACP, and it is now known that osteopontin could bind to ACP and prevent its transformation to HAP (31). Thus, matrix proteins are responsible for regulating the transformation of amorphous calcium phosphate to crystalline hydroxyapatite as well as stabilizing the ACP phase when required. Overall matrix intervention during the nucleation and growth process may give rise to composite materials like bone and dentin with functionalized mechanical properties.

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