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Self-Assembled Collagen−Apatite Matrix with Bone-like Hierarchy

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Bone tissue strength depends, aside from its cellular components, on its density (ratio mineral vs organic phase), its matrix composition (mainly hydroxyapatite (HA) and collagen), its overall structure (microarchitecture of compact and trabecular compartments), and its rate of remodeling. Microarchitecture is the most important criterion used by surgeons to assess bone quality as well as normal mechanical functionality[1,2] and is, thus, extensively studied in tissue engineering.[3] A three-dimensional, hierarchical organization over many length-scales (nano-, micro-, millimeters and more) characterizes this particularly complex composite material.[4] Indeed, "mineralized collagen fibrils" organize into bundles to form fibers ("fibril arrays") that further pack in regularly dense and ordered networks[5] ("fibril array patterns"). Another essential characteristic is the coalignment between the crystallographic *c*-axes of the hydroxyapatite nanocrystals and the long axes of the collagen fibrils.[6,7]

Consequently, mimicking bone is a tremendous challenge and the aim for many in the fields of materials science and tissue engineering, especially since the design of such hybrid bio-organic/inorganic materials requires mineralization processes compatible with the organic components. This is particularly true in the presence of collagen, the protein being irreversibly denaturated into gelatin with heating, then losing its self-ordering properties. Several groups have attempted to prepare bone-like materials from organic and mineral constituents.[8−14] Most of these studies have successfully replicated an essential characteristic in bone, which is the predominant coalignment of the organic and mineral phases. However, according to the Weiner and Wagner terminology,[4] experiments have only been able to organize such structures up to the fibrillar level (∼100−300 nm). Actually, in all the aforementioned studies, the final collagen concentration is well below 3 mg/mL, preventing formation of a defined three-dimensional collagen fibrillar architecture similar to that found in compact bone.[15] Moreover, one has to notice that the absence of fibril banded patterns in certain studies suggests a mineralization process incompatible with physiological fibrillogenesis.

Here, we report the preparation of a collagen−apatite matrix, enabling organization of collagen fibrils into 3D scaffolds and, concomitantly, allowing nucleation and coalignment of HA crystals within the matrix from the nano- to millimeter scales. The process is based on a "one-pot" coprecipitation method at room temperature coupling the liquid-crystalline properties of collagen[16] to a HA mineralization process.[17] Indeed, the ability to organize collagen molecules at unusually high collagen concentrations through liquid crystalline ordered phases has been reported,[16] along with the possibility to then stabilize the viscous phases in 3D fibrillar matrices.[18] The coprecipitation step of both the calcium-phosphate salts and pure collagen monomers extracted from rat tail tendons[19] (300 mg/mL) was initiated through increase of pH by ammonia gas diffusion. An acidic polymer, polyaspartate, was added to the dialyzed collagen solution to mimic the role of specific soluble proteins.[20,21]

Synchrotron X-ray diffraction with a 50 μm beam size was used to study the collagen−apatite matrix sample by averaging over larger length-scales compared to electron

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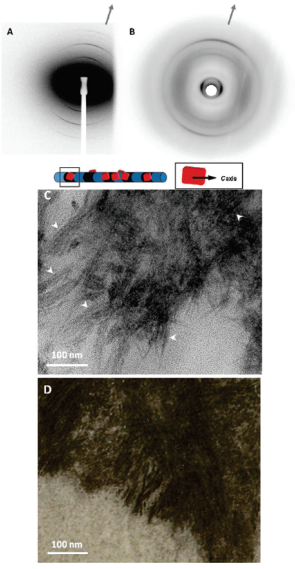

Figure 1. The "mineralized collagen fibril" level (~100−300 nm) (scheme) demonstrated by small-angle X-ray scattering (SAXS), wide-angle X-ray diffraction (WAXD), and transmission electron microscopy (TEM). (A) SAXS pattern of a mineralized collagen matrix. Type I collagen fibrils are revealed by the equidistant reflections arising from the 67 nm axial periodicity. The strong anisotropic scattering indicates preferential alignment of the fibrils in the matrix. (B) WAXD pattern of the mineralized matrix recorded on a flat film. The presence and orientation of the (002) diffraction peak of HA indicate the coalignment of the crystallites *c*-axis and the fibrils long axes. (C) TEM micrograph of an ultrathin unstained section of the above collagen−apatite matrix. HA crystals are observed in parallel arrays within collagen fibrils (white arrows). (D) Similar observations in partly demineralized human bone.

diffraction, which only provides local material characterization due to the small beam size (< 1 μm).[8−14] The sample revealed a heterogeneous organization with the coexistence of aligned and isotropic scattering patterns as similarly observed in bone.[22] Uniformly oriented domains at least 50 μm large were observed. A typical 2D small-angle X-ray scattering (SAXS) pattern of the mineralized matrix in such domains showed several equidistant reflections arising from the 67 nm axial periodicity, characteristic of collagen fibrils (Figure 1A). The strong anisotropic scattering revealed the spontaneous alignment of the main fibril axes over large distances. Such alignment was also observed in SAXS studies of partially demineralized bovine compact bone (see Supporting Information Figure 1). Wide-angle X-ray diffraction (WAXD) was used to study simultaneously the organic and mineral phases (Figure 1B). Several diffraction peaks can be assigned to the characteristic HA (002) and weaker (211), (300), (202) diffraction lines. Furthermore, characteristic collagen reflections were observed, that is, equatorial at $q = 2\pi/(1.3\ \text{nm})$ and meridional at $q = 2\pi/(0.29\ \text{nm})$, corresponding to the lateral packing of the collagen

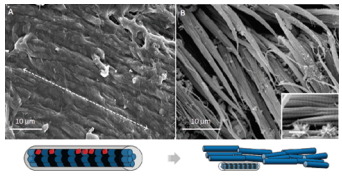

Figure 2. "Fibril array" level (~1−3 μm) illustrated by SEM. (A) Fibers result from the parallel packing of the fibrils long axes (scheme) and are oriented along a preferential direction (~10−100 μm) (white dotted line). The HA crystals, intimately embedding the closely packed collagen fibers (mean diameter of ~2 μm), give a smooth appearance to the sample. (B) The matrix obtained by coprecipitation without polyaspartate leads to individual spherulitic HA crystals which are located beside the collagen network. (Inset) Bundles of fibrils are observed with typical cross-striated pattern (scale bar 1 μm).

molecules and to the distance between amino acids in the polypeptide chain within oriented collagen fibrils, respectively.[23−25] This pattern was similar to that in partially demineralized fish bone.[26] Since the mineralization degree was close to that in very early stages of normal in vivo calcification,[7] the resulting degree of mineralization was low enough to observe the collagen signal (see Supporting Information Figure 2). It was indeed found to be ~6% by thermogravimetric analysis (Supporting Information Figure 3) In most domains, the diffraction anisotropy indicated a preferential orientation of the HA crystallites in the matrix, in agreement with previous investigations of the apatite crystallographic orientation in bone. The orientation of the HA (002) reflection was parallel to the 67 nm collagen reflection, which demonstrated that the crystalline *c* axes and the axial direction of the fibrils were aligned. The X-ray diffraction data are supported by transmission electron microscopy (TEM). Observations of the collagen−apatite matrix (Figure 1C) shows that the crystallites were indeed located within the collagen fibrils since observations were performed on ultrathin sections. A preferential parallel orientation is similar to observations in partially demineralized human bone (Figure 1D). In contrast, in the absence of collagen fibers, HA crystallites did not organize in parallel arrays and their sizes were significantly larger (see Supporting Information Figure 4), emphasizing the important role of confinement within the dense matrix in bone. Consequently, the "mineralized collagen fibril" level (~100−300 nm) is reached here.

Scanning electron microscopy (SEM) of the collagen−apatite matrix revealed the preferred parallel orientation of the collagen fibrillar long axes at the micrometer level and an apparent homogeneous apatite coating, as shown in Figure 2A. Indeed, in the absence of polyaspartate, cross-striated fibers (diameter ~ 1−3 μm) are revealed together with homogeneously nucleated HA spherulitic crystals (diameter ~ 1 μm) (Figure 2B and inset). Thus, it confirms the mineralization process biocompatibility and emphasizes the role of polyaspartate in driving mineral ions.[13,20,21] These results complement conclusions obtained by solid-state NMR investigations of the protein−mineral interface in bone whereby HA crystallites were

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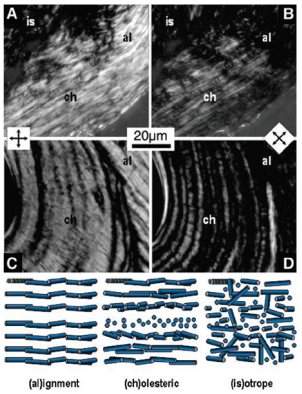

Figure 3. "Fibril array patterns" level (~0.1−1 mm) illustrated by polarized light microscopy, is characterized by a spatial coexistence of different domains, i.e., isotrope, aligned (nematic), and twisted (cholesteric) (scheme). Thin sections of collagen−apatite matrix (A, B) and human bone (C, D). Both samples exhibit large birefringent domains (left side) which become darker after a 45° rotation of the polarizers (right side). Thin alternating bright and dark bands reveal the twisted plywood organization of collagen fibers (ch). Uniformly bright or dark domains correspond to parallel dense packing of collagen fibers (al). Domains always remaining dark correspond to random distribution of collagen fibrils (is).

assumed to interact with noncollagenous components of the protein matrix.[27] Interestingly, the present process gives access to the "fibril array" level (~1−3 μm).

Polarized light microscopy was performed on thin sections of collagen−apatite matrices providing evidence of a material oriented at the millimeter scale by observing the birefringence of large domains (Figure 3A and Supporting Information Figure 5A). Additionally, isotropic domains can be revealed where no birefringence was observed. Some domains showed thin alternating bright and dark bands which reveal the twisted plywood organization (i.e., cholesteric) of the collagen fibers. This plywood motif can be tracked by rotating the sample stage and following the extinction of light specifically in areas where the collagen molecules lie parallel to the direction of the polarizers (Figure 3B and Supporting Information Figure 5C). Moreover, uniformly bright or dark domains corresponded to parallel dense packing (i.e., aligned) of collagen fibers. The birefringent extinction properties were similar to that observed in osteons of human compact bone (Figures 3C,D). The variability and heterogeneity of the oriented domains were observed both in the synthetic matrix and native bone tissue.[22,7,28] In summary, light microscopy data confirm that the collagen−apatite matrix displays the long-range organization described in bone, that is, the "fibril array patterns" level according to the previously proposed terminology by Weiner and Wagner.[4]

The mechanical behavior of the collagen−apatite matrix was investigated by nanoindentation. At first glance, the modulus of the collagen−apatite matrix was found to be 6.49 ± 1.87 GPa (mean ± std.dev.), close to the collagen matrix (without HA) with 4.65 ± 0.90 GPa, whereas fibrolamellar bone was 23.11 ± 4.18 GPa.[1] Despite these differences, significant attention should focus on the similar degrees of mechanical anisotropy observed in the collagen−apatite matrix and in fibrolamellar bone; the moduli at 0° and 90° were significantly different ($p < 0.05$) with a decrease from the transverse (6.5 GPa) to the longitudinal section (4.3 GPa) by about 50%, a ratio very similarly to what was previously found in bovine bone[1] (see Supporting Information Figure 6). This comparison of mechanical anisotropy further confirms that the apatite and collagen in the matrix are coaligned, and in a similar manner to which exists in bone. The differences in moduli between the matrix and fibrolamellar bone can be attributed to the relative differences in mineralization (~6% in the matrix vs ~50% in native bone). Interestingly, the lower mineralized bone mimetic matrix possesses better mechanical properties than a diluted collagen matrix which is fully mineralized,[29,30] providing evidence that matrix microarchitecture is essential to assess mechanical performance and, subsequently, bone quality.

The mineralized matrix reported here has characteristic multiscale bone-like hierarchy. We show that the dense and ordered collagen matrices together with a soft HA precipitation process are key factors for mimicking bone microarchitecture. These results emphasize the role of physicochemical processes in biomineralization events that are most often discussed only from the viewpoint of biological control. The actual mineral content of the collagen−apatite matrix is, however, much lower than in fully developed bone. Nevertheless, bone development in vivo starts as a nearly unmineralized osteoid tissue that is gradually mineralized leading to a characteristic inhomogeneous mineralization pattern.[31] Thus this bone mimetic matrix should also appear to be an appropriate substrate to study the behavior of bone cells in vitro.[32]

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Supporting Information Available: Experimental details and the following graphics: SAXS pattern, WAXD patterns, TG and DTG analysis, TEM micrographs, polarized light microscopy, elastic moduli, and other images (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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