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MINERALIZATION OF AN AXIALLY ALIGNED COLLAGENOUS MATRIX: A MORPHOLOGICAL STUDY

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Abstract

Bone can be described as a highly ordered composite of type I collagen integrated with an inorganic mineral phase. In vitro models of bone mineralization using collagenous substrates have been reported in the literature. This study reports an in vitro system of mineralized reconstituted collagen fibers, with aligned fibrillar substructure. The collagen fibers were mineralized in a double diffusion chamber saturated with respect to calcium and phosphate. The morphology and ultrastructure of the mineral precipitate were evaluated as a function of the pH of the incubating media. Brushite crystal was observed at acidic pH. Large rectangular crystals formed at pH 5.15 and appear to associate with the collagen fibers. At neutral and alkaline pHs, hydroxyapatite crystals were observed in association with the collagen fibers. Spherical aggregates of hydroxyapatite crystals were seen at neutral and alkaline pHs, but these structures were reduced in size when formed on collagen at alkaline pH. On close examination these spherical structures were found to be hollow when viewed in cross section.

The crystals precipitated within the interior of the collagen fiber at neutral and alkaline pHs were comparable in both size and shape to crystals observed in mineralized turkey tendon and skeletal tissues. These preliminary observations indicate that with further refinement the reconstituted collagen fibers may prove useful in model systems for the study of collagen mediated mineralization in vitro. In addition, mineralization of collagenous matrices may lead to the development of biomaterials for bone repair and replacement.

Key Words: Mineralization, reconstituted type I collagen, crystal morphology, pH, scanning electron microscopy, transmission electron microscopy, electron diffraction.

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Introduction

Bone formation involves osteoblasts derived from undifferentiated mesenchymal cells or differentiated chondrocytes [36] that synthesize a highly ordered collagenous matrix named osteoid. A direct association between the inorganic and organic phases of mineralized tissue was demonstrated by early ultrastructural studies [18, 35].

Matrix mineralization in bone is confounded by the complex hierarchical structure of this tissue and therefore other models have been studied. The mineralized turkey leg tendon is structurally similar to other hard tissues [2], but due to the high degree of axial orientation of the collagen matrix, it is useful for both biophysical [41] and ultrastructural investigations [30]. Arsenault [3] and Landis [30,31] have separately identified the events in turkey tendon mineralization. In general, Arsenault [3] observed mineral localized within membrane bound vesicles. Mineral spreads from the vesicles to the adjacent collagen fibrils, and is eventually deposited interfibrillarly. Mineralization progresses in this manner both axially and laterally. Landis and Song [31] recently concluded from a study of mineralized turkey using high voltage electron microscopy and 3D image reconstruction, that vesicle mediated mineralization is dispersed throughout the tendon, and the bulk of mineralization is collagen mediated. In addition, vesicle and matrix mineralization were seen to appear at spatially and structurally distinct sites.

A feature commonly observed between the inorganic phase and the type I collagen fibril, has been the preferential orientation of the mineral c axis with the axis of the collagen fibril [19]. Heywood et al. [21] in a recent study utilizing high resolution transmission electron microscopy have identified a high degree of specificity in the crystal association with respect to the collagen fibril and conclude this is indicative of a direct interaction between the organic matrix and the embryonic crystal during nucleation.

Because of this distinct spatial association between the inorganic phase and the fibrillar structure of the collagen molecule, a number of

acellular, in vitro studies of matrix mediated mineralization have been conducted. These studies have focused on the isolated influence of type I collagen or the variety of non-collagenous proteins associated with the extracellular matrix of bone [22]. For a review of the non-collagenous proteins associated with matrix mineralization (see Boskey [13]). Tables 1A and 1B summarize in vitro studies of collagen mediated mineralization. The in vitro models can be categorized as fibrillar systems or as gel systems [22]. The majority of these in vitro systems consist of salt or heat precipitated acid soluble type I collagen with randomly oriented fibrils calcified in metastable solutions of calcium and phosphate.

In reviewing these reports, it is apparent that few of these papers integrate biophysical studies with morphological and ultrastructural analyses. Also note worthy is the absence of the pH dependency of collagen mediated mineralization. These studies suggest that type I collagen is found in general to participate in mineralization, but the exact role that it plays is still undefined [19, 22].

In order to understand more concerning the possible role of type I collagen in mineralization, we have developed an in vitro system consisting of reconstituted collagen fibers which were exposed to supersaturated solutions of calcium and phosphate at acidic, neutral, and alkaline pH in a double diffusion chamber [14]. The system in this study is a modification of the double diffusion model developed by Boskey [12]. The crystal morphology and ultrastructure of the mineral formed within the collagen fibers were observed as a function of the pH of the incubating media. Our results suggest that this aligned fibrillar substructure may provide a template for mineralization in a manner comparable to turkey tendon mineralization observed in vivo.

Materials and Methods

Production of reconstituted collagen fibers

Reconstituted collagen fibers were produced via the methods presented by Kato et al. [26]. Standard SDS - PAGE and amino acid analysis were employed to establish the purity of the collagen isolated from bovine corium used in this study [26]. Based on these analyses, the material was found to be purely type I collagen with no evidence of noncollagenous protein contamination. A 1% w/v solution of type I collagen in distilled water at acid pH (pH 2.0) was blended at high speed for 4 minutes to produce a collagen dispersion. This dispersion was then degassed under a high vacuum to remove air introduced during the blending process. The evacuated dispersion was then extruded through thin polyethylene tubing (I. D. 0.5mm, P.E. 50 tubing, Clay Adams, Parsipanny, NJ) into a fiber formation buffer (FFB) at pH 7.4 and 37° C [17]. The FFB was produced by combining 135 mM NaCL, 30 mM TES (N-tri(hydroxymethyl) methyl - 2 - aminoethane sulphonic acid) and 30 mM sodium phosphate dibasic in distilled water. Fibril formation was allowed to proceed in the FFB for 45 minutes, at which time the solution was aspirated and replaced with isopropyl alcohol and allowed to set for 4 hours. Finally, the alcohol was replaced with distilled water for 10 minutes and the fibers removed and air dried under tension overnight. Prior to use, the fibers were stored in sealed containers with a small quantity of dessicant.

Collagen fiber mineralization

Mineralization was conducted in a specially prepared double diffusion chamber [14]. The chamber consists of two separate ion reservoirs, with each reservoir containing 4 liters of solution. The first reservoir contained a Tris buffered solution of calcium chloride (0.05 M Tris and 0.1 M calcium chloride) and the second chamber contained a Tris buffered solution of potassium phosphate (0.05 Tris and 0.1 M potassium phosphate). The reservoirs were separated by a small ion permeable membrane of dialysis tubing (mol. wt. cut-off 12,000 - 14,000, Spectra/Por 4 Dialysis Membrane Tubing, Los Angeles, CA). Within the envelope of the dialysis tubing, a parallel array of 10 - 15 collagen fibers was mounted on vellum paper tabs with quick drying epoxy. To this was added approximately 5 - 10 ml of 0.05 M Tris buffered solution. Prior to the addition of the solutions to the appropriate compartments, the pH of each was adjusted with 6 N HCL to form solutions with pHs ranging from acidic (pH 5.0) to alkaline values (pH 10.0). The poor buffering capacity of Tris at the acidic pH points required careful addition of HCl to reach the lower pH values. Due to the concomitant decrease in pH following calcium phosphate precipitation [11], the pH of the solution within the dialysis membrane where precipitation occurs, was measured at the end of the 7 day incubation period to more accurately reflect the pH of mineralization. After the initial adjustment and chamber set-up, the solutions were allowed to equilibrate, and no attempts were made to maintain pHs at the starting levels. Following incubation, the mineralized collagen fibers were removed from the vellum paper tabs and allowed to air dry overnight before processing for light, scanning and transmission electron microscopy.

Upon assembly of the chamber and addition of the respective solutions, precipitation was noted to occur within 2 - 5 minutes inside the dialysis membrane window separating the two ion reserviors. The quantity of mineral precipitate which forms inside the membrane appears to reach a constant level within 1 hour of the start of each experiment. Solute concentrations were not monitored, and the individual ion reservoirs were checked at the end of each experiment for evidence of precipitation indicating cross contamination between the ion reservoirs.

Table 1A: Summary of In Vitro, Acellular, Collagen Mediated Mineralization: Fibrillar Systems.

Reference and Date	Matrix Source, Preparation and Properties	Methods of Mineralization	Analysis Techniques	Results
Glimcher et al. [20] (1957)	Type I collagen, (source: bovine amd guinea pig skin). Temperature induced fibril formation. Matrix = random fibrils	Metastable solution of calcium and phosphate, neutral pH	X-Ray diffraction T.E.M. Electron diffraction Analytical chemistry	Native type I collagen fibrils required for the formation of apatite crystals from metastable solutions.
Bachra & Sobel [5] (1959)	Type I collagen, (source: rat skin and tail tendon). Fibrils salt precipitated from solution Matrix = random fibrils	Metastable solution of calcium and phosphate, neutral pH	Analytical chemistry	Reconstituted acid soluble collagen with and without chondroitin sulfate, heparin laryl sulfate NaCl , calcified in mineralizing solutions.
Bachra & Fisher [6] (1968)	Type I collagen, (source: rat tail tendon). Salt precipitated from solution. Matrix = random fibrils Type I collagen, (source: sheep bone) Fine particles of demineralized bone matrix	Metastable solution of calcium and phosphate, neutral pH	Analytical chemistry	Demineralized sheep bone collagen a potent nucleator of apatite and collagen extracted form rat tail tendon a poor nucleation catalyst.
Wadkins [38] (1968)	Type I collagen, (source: bovine tendon). Two extraction techniques, with and without KCl extraction step. Matrix = particles of fibrils and fibril bundles.	Metastable solution of calcium and phosphate, neutral pH	Analytical chemistry Light Microscopy, Von Kossa staining.	Ion uptake nearly identical for both extractions, only tendon extracted without the KCL step exhibited Von Kossa reaction.
Katz [27] (1969)	Type I collagen, (source: bovine skin). Matrix = random fibrils	Metastable solution of calcium and phosphate, neutral pH	Analytical chemistry	Mineral uptake by 640Å periodic collagen takes place by nucleation and crystal growth. The rate is diffusion limited. Collagen reduces the size of crystal nucleus necessary for mineralization.
Jethi et al. [24] (1970)	Type I collagen (source: bovine tendon). Matrix = small aggregates of fibrils	Metastable solution of calcium and phosphate, neutral pH	Analytical chemistry	Collagen induces mineral formation, and calcium or phosphate ion exchange with mineralized collagen requires alternate ion in solution.
Jethi & Wadkins [25] (1971)	Type I collagen (source: bovine tendon). Matrix = small aggregates of fibrils	Metastable solution of calcium and phosphate, neutral pH	Analytical chemistry	Propose multi-step process for collagen mediated mineralization
Bachra [4] (1972)	Type I collagen, (source: Rat tail tendon). Salt precipitation from solution. Matrix = random fibrils Type I collagen (source: sheep bone). Particles of demineralized bone matrix	Metastable solution of calcium and phosphate, neutral pH	Analytical chemistry	Rat tail tendon collagen a poor nucleator of mineral, and sheep bone collagen a potent nucleator. Mineral forms in vitro occurs in two separate phase.
Koutsoukos & Nancollas [29] (1987)	Type I collagen, (source: bovine tendon). Matrix = particles of fibril bundles	Supersaturated solution of calcium and phosphate, neutral pH.	Analytical chemistry S.E.M.	Confirm induction of hydroxyapatite nucleation and growth on collagen at low supersaturation.
Clarke et al. [15] (1991)	Type I collagen, (source: rabbit bone). Matrix = cubes of demineralized bone matrix	Supersaturated solution of calcium and phosphate, at pH (3.5 - 5.4)	X-Ray microprobe analysis Crystallographic analysis	At low pH, tricalcium phosphate (TCP) forms carpet covering collagenous matrix, and at increased pH brushite crystals grew over the TCP surface.

Table 1B: Summary of In Vitro, Acellular, Collagen Mediated Mineralization: Collagen Gel Systems.

Reference and Date	Matrix Source, Preparation and Properties	Methods of Mineralization	Analysis Techniques	Results
Pokric & Pucar [34] (1979)	Type I collagen, (source: rat tail tendon) Salt and heat precipitation of collagen gel. Matrix = random fibrils	Double diffusion. Ion reserviors of calcium and phosphate at neutral pH.	Analytical chemistry	Critical concentration of mineral formation was not affected by any of the gel systems evaluated. Collagen does not promote mineralization.
Hunter et al. [23] (1985)	Type I collagen, (source: rat tail tendon) Precipitate of collagen fibrils Matrix = random collagen fibrils	Gel containing collagen fibrils and saturated with phosphate ions, covered with solution saturated with calcium ions.	Analytical chemistry X-Ray diffraction	Hydroxyapatite crystal growth at the solution/gel interface, study determined inhibitory effect of chondroitin sulfate
Brecevic et al. [9] (1987)	Collagen gelatin, (source: bovine skin) Matrix = degraded collagen	Equi-molar concentration of calcium and phosphate at neutral pH	Particle electrophoresis pH, and turbidity	Gelatin slows or prevents ACP aggregation and promotes transformation of ACP to crystalline phase, (ACP = amorphous calcium phosphate).
Boskey [12] (1989)	Type I collagen, (source: lathyritic rat skin) Matrix = random fibrils in gel substrate	Double diffusion. Ion reserviors of calcium and phosphate at neutral pH.	Analytical chemistry X-Ray diffraction T.E.M.	Fibrillar collagen reduced the time to formation of hydroxyapatite within the gel system, conclude collagen promotes mineralization.
Blumenthal et al. [8] (1991)	Type I collagen gel Matrix = random fibrils	Double diffusion. Ion reserviors of calcium	Analytical chemistry FT-IR	Type I collagen exerts an inhibitory effect on the proliferation of hydroxyapatite.

Light microscopy

Control and mineralized collagen fibers were viewed under conventional and polarized light microscopy with a Lietz Laborlux Pol 12 light microscope (Ernest Lietz, Wetzler, Germany). Diameters of the fibers in the dry and hydrated state were measured with a calibrated eyepiece and a 10X objective.

Scanning electron microscopy

The morphology of control reconstituted collagen fibers and mineralized collagen fibers was analyzed via scanning electron microscopy using the methods reported by Wasserman et al. [39]. Air dried fibers (15 fibers from 3 separate experiments at each of the pH points studied) were mounted on aluminum stubs with a conducting silver paste (Ted Pella Inc. Redding CA). The specimens were then sputter coated with a thin layer of gold (40 nm) and viewed on an Amray 1400 at an operating voltage of 20 kV.

Transmission electron microscopy

Control collagen fibers were hydrated overnight in 0.05 M Tris buffer prior to fixation in modified Karnovsky's fixative (2% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate and 4 mM $CaCl_2$ at pH 7.4) overnight. The fibers were then washed in 0.1 M cacodylate and 1.5 mM $CaCl_2$ buffer and post fixed with 1 % OsO_4 and 1.5 mM $CaCl_2$ for 1 hour. Post fixation was followed by a wash in cacodylate (0.1 M) and then dehydration through serial solutions of methanol from 30% to 100% and finally infiltrated and embedded in Spurr Embedding Media (Ted Pella Inc. Redding CA). The embedded specimens were sectioned in directions perpendicular and parallel to the axis of the fiber and collected on formvar coated slot grids. Mineralized and control collagen fibers collected on slot grids were double stained using 2% aqueous uranyl acetate and lead citrate.

Air dried mineralized fibers were embedded directly in Spurr's embedding media (Ted Pella Inc., Redding CA) following an extended infiltration schedule. Blocks of the material were rough trimmed and thin sections (~ 100 - 120 nm) cut with a diamond knife were obtained in directions perpendicular axis of the fiber. The sections were floated briefly on water (pH 8.5) and collected on formvar coated slot and mesh grids. All sections were viewed unstained. Selected area electron diffraction patterns of mineral formed within the collagen were obtained from unstained longitudinal sections of the mineralized collagen fibers. The selected area was defined by a 150 μm diffraction aperture. The camera constant of the electron microscope in the diffraction mode was calibrated using a known sample of evaporated aluminum.

The lattice constants of the crystals observed within the mineralized collagen fibers formed at neutral and alkaline pH were calculated and compared with X-ray powder diffraction data files (JCPDS-ICDD © 1989) for a variety of calcium phosphate minerals. The large crystalline structures formed at acidic pH embedded poorly in Spurr's epoxy, and were considered too large and dense a material for thin sectioning with a diamond knife.

Results

Light microscopy

Dry reconstituted collagen fibers examined under light microscopy appeared as thin (44.4 ± 2.3 μm) strands of opaque material with an overall rough surface morphology. Upon rehydration, the fibers swelled to an average diameter of 127.6 ± 5.2 μm. When viewed under polarized light (Figure 1a), there was evidence of a mild extinction pattern seen as a dark to light banding along the length of the fiber.

Scanning electron microscopy

The surface morphology of the control collagen fibers exhibited a rough exterior with undulations in the fiber diameter along its length (Figure 1b) and ridges and crevices were also apparent running roughly parallel to the axis of the fiber. These structures are attributed to the fibrillar substructure of the reconstituted collagen fiber.

Review of scanning electron micrographs of the mineralized fibers formed at pH 4.65 and pH 5.15 show two general crystal morphologies. Crystal precipitates formed either large flat plate-like structures or appeared as stellate aggregates of smaller crystals. In low magnification micrographs of mineralized fibers formed at pH 4.65, the plates appear to radiate off of single nucleation sites along the axis of the fiber (Figure 2a). At higher magnification, the plates appear to be composed of individual layers formed one on top of the other (Figure 2b). Mineral formed at pH 5.15 (Figure 2c) had a similar gross morphology, specifically the presence of large flat rectangular plates, or stellate aggregates of smaller crystals (Figure 2d). The large rectangular plates formed at pH 5.15 often appeared oriented with respect to the collagen fiber axis.

Scanning electron microscopy of mineral on the surface of collagen fibers at pH 7.0 revealed a fairly uniform surface coating composed of thin rectangular crystals, as well as clusters of these crystals arranged in spheres (Figures 3a and 3b). Measurements of these spherical structures indicate an average diameter of 9.8 ± 3.5 μm (n=30). At the alkaline pH, 8.4, the mineral crystals formed in the shape of small spheres which were dispersed over the surface of the fiber (Figures 3c and 3d). Measurements of these small spherical structures

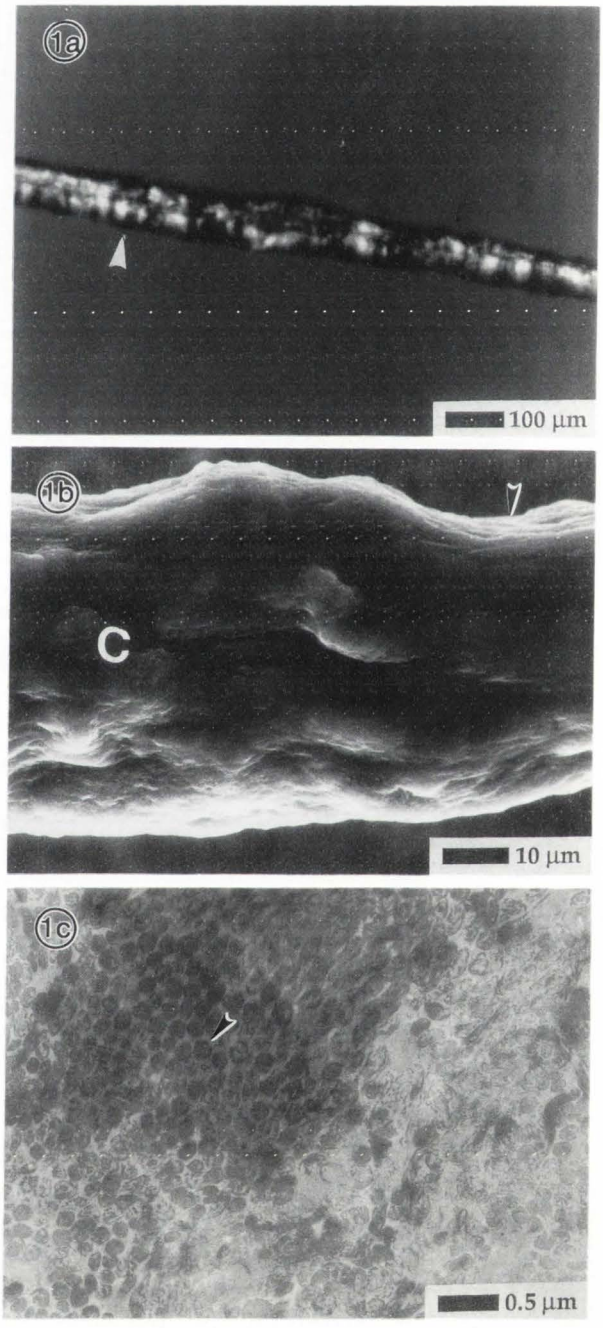

Figure 1: Light and Electron Micrographs of Control Collagen Fibers. Figure 1a is a representative polarized light micrograph of the control collagen fiber, showing a mild extinction pattern (arrow indicates a region of distinct banding). Figure 1b depicts the typical features of the collagen fiber (C) seen under scanning electron microscopy. The arrow indicates evidence of fibrillar substructure. Figure 1c shows a transmission electron micrograph of a section perpendicular to the fiber axis (arrow indicate collagen fibrils seen in cross section).

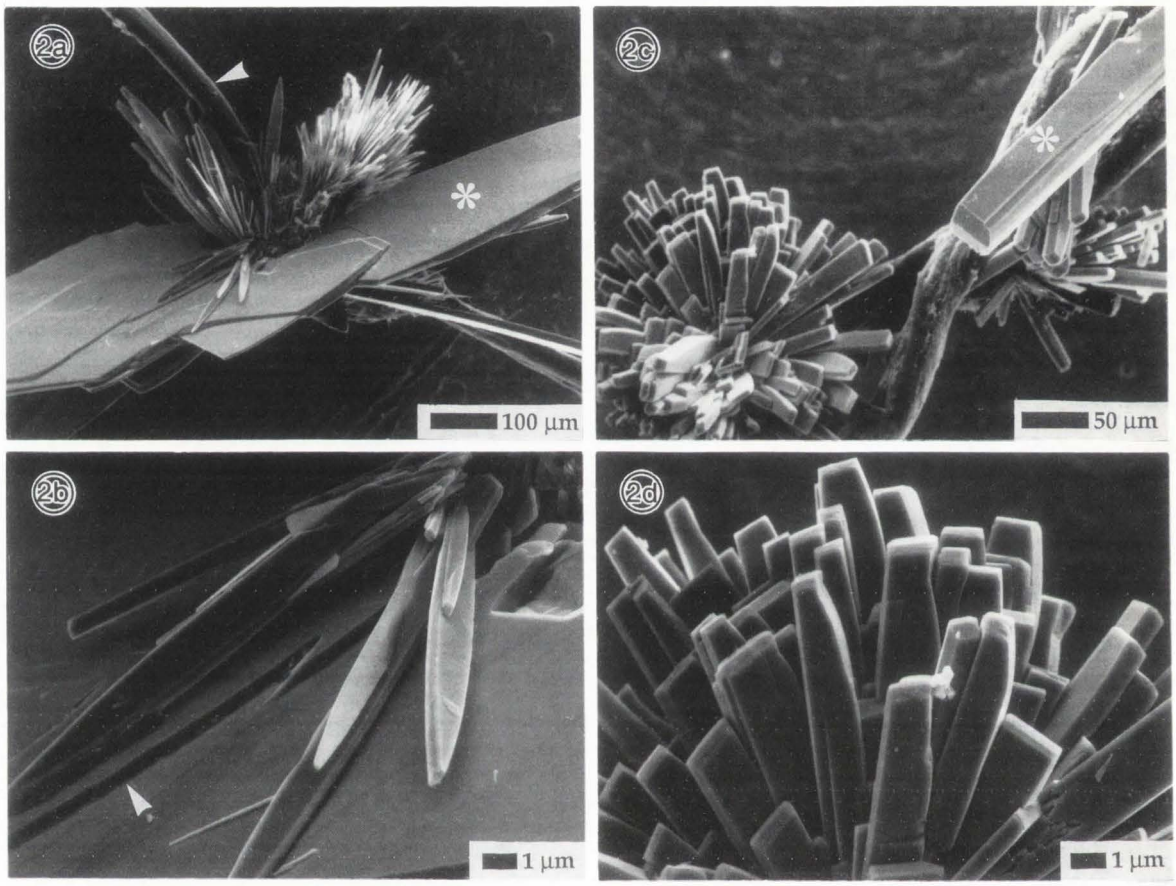

Figure 2: Scanning Electron Microscopy of Mineralized Fibers Formed at Acidic pHs. Figures 2a and 2b show low and high magnification views of mineralized collagen formed at pH 4.65. In figure 2a the collagen fiber is indicated by an arrow. Radiating off of a single nodule on the fiber are two separate crystal structures, 1) large plate-like structures (*) and 2) stellate aggregates of fine needle-like crystals. Figure 2b indicates a step (arrow) in the plane of the large crystalline plate. Figures 2c and 2d present micrographs of the mineral formed on collagen fibers at pH 5.15. Two crystal morphologies are evident in figure 2c, specifically large axially oriented rectangular rods (*) and stellate aggregates of long thin rectangular crystals. Figure 2d is a higher magnification view of the stellate aggregates.

indicated an average diameter of 1.4 ± 0.4 μm (n=30). A statistical comparison between the diameters of the spherical structures formed at neutral and alkaline pHs indicated that the structures formed at alkaline pH were significantly smaller ($p \leq 0.05$ "Students" t test) than those seen at neutral pH.

Transmission Electron Microscopy

Micrographs obtained from sections perpendicular to the axis of the control collagen fibers indicate the majority of the collagen fibrils were seen in cross section, with an average fibril diameter of 129.5 ± 31.0 nm (Figure 1C). Regions were noted in each transmission electron micrograph where collagen fibrils were seen oriented perpendicular to the axis of the fiber, but the bulk of the fibril substructure appeared to be oriented parallel with the axis of the fiber.

Transmission electron micrographs of unstained sections, obtained from cross sections of mineralized collagen fibers indicated the formation of mineral crystals throughout the interior of the fiber and the formation of spherical structures on the surface of the fiber. At both pH 7.0 (Figure 4a) and pH 8.4 (Figure 4b) the crystalline spheres seen in cross-section appeared as hollow arrays of small plate-like crystals. Figure 4c shows the features of the mineral crystal formed within the interior of the collagen fiber. The flat plates were generally rectangular in shape and approximately 95 nm X 53 nm in length and width respectively.

Figure 4d shows a characteristic electron diffraction pattern obtained from unstained sections of the mineralized fiber formed at neutral and alkaline pHs. The diffraction pattern is representative of a polycrystalline, or randomly

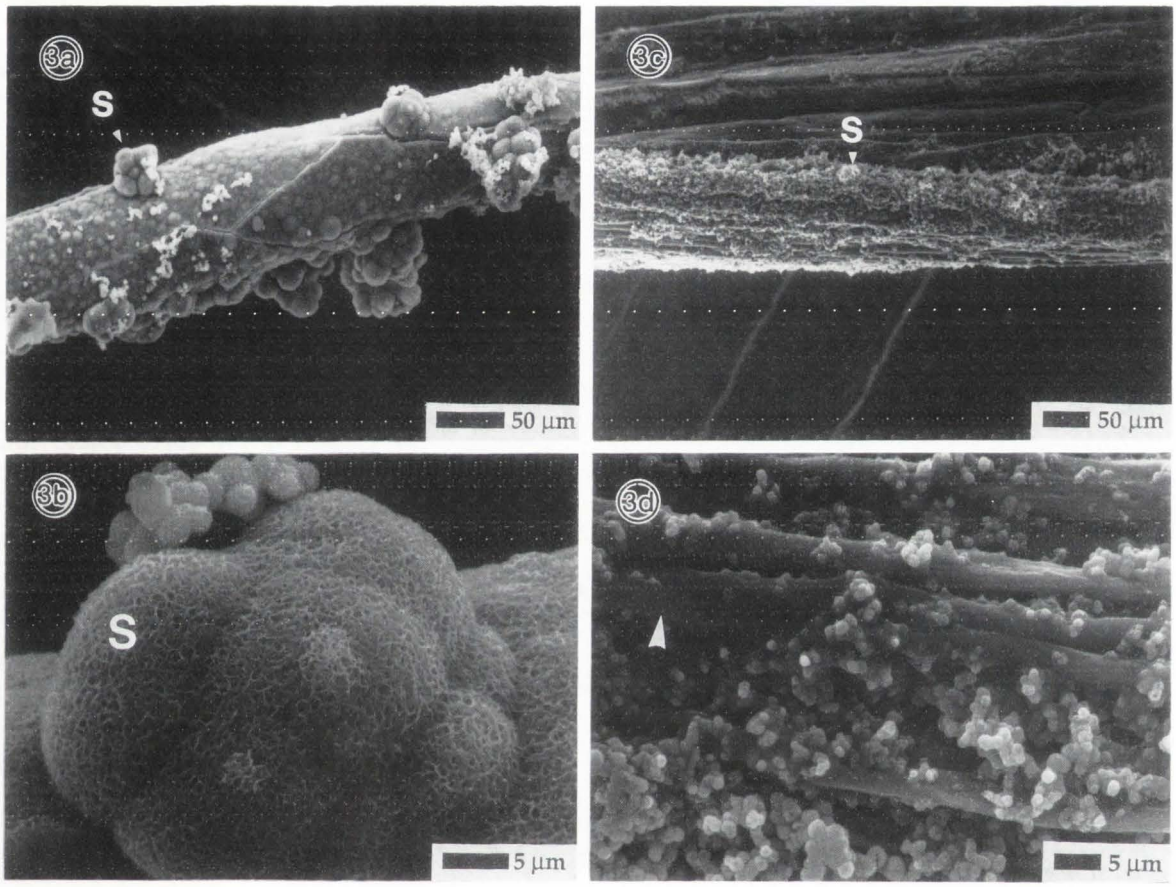

Figure 3: Scanning Electron Microscopy of Mineralized Fibers Formed at Neutral and Alkaline pHs. The micrograph in figure 3a indicates a uniform surface coating of mineral precipitate on the collagen fiber formed at pH 7.0, with the addition of spherical crystalline aggregates (S) dispersed over the surface of the fiber. At higher magnification (3b) the crystalline spheres (S) appear as clusters of small plates. Figure 3c defines the surface morphology of the mineral formed on collagen fibers at pH 8.4. The precipitate consists of small spherical structures (S) spread evenly over the surface. Figure 3d shows these structures in greater detail (arrow indicates fibrillar substructure in the fiber).

oriented sample of hydroxyapatite crystals based on comparison with the X-ray powder diffraction data files for hydroxyapatite.

Discussion

The observance of an extinction pattern within fibrous collagen tissue seen under polarized light arises from a microscopic scale periodicity in the structure which is described as a regular crimping of the fibril along the axis of orientation [16,17]. Reconstituted collagen fibers observed under polarized light indicated distinct regions with extinction patterns. The fibers were analyzed with a polarized microscope equipped with a rotating stage, and the periodic regions were observed to move continuously along the length of the fiber with rotation of the stage. This implies that the periodicity does not arise from irregularities or structural discontinuities in the surface [17], but, it is important to note that this phenomena was isolated to specific regions within the fiber. Combination of the results from polarized light microscopy and transmission electron microscopy imply that the fibrillar substructure of the reconstituted collagen fiber is aligned with the fiber axis. Exact determination of the axial alignment of these fibrils would require biophysical investigations involving possibly X-ray, electron, or optical diffraction techniques. These observations corroborate results obtained by Wasserman et al. [39] from a similar reconstituted collagen fiber.

The morphology of the crystals formed in association with the collagen fibers mineralized at pH 4.65 are comparable to brushite crystals observed to form in association with casein [37] and

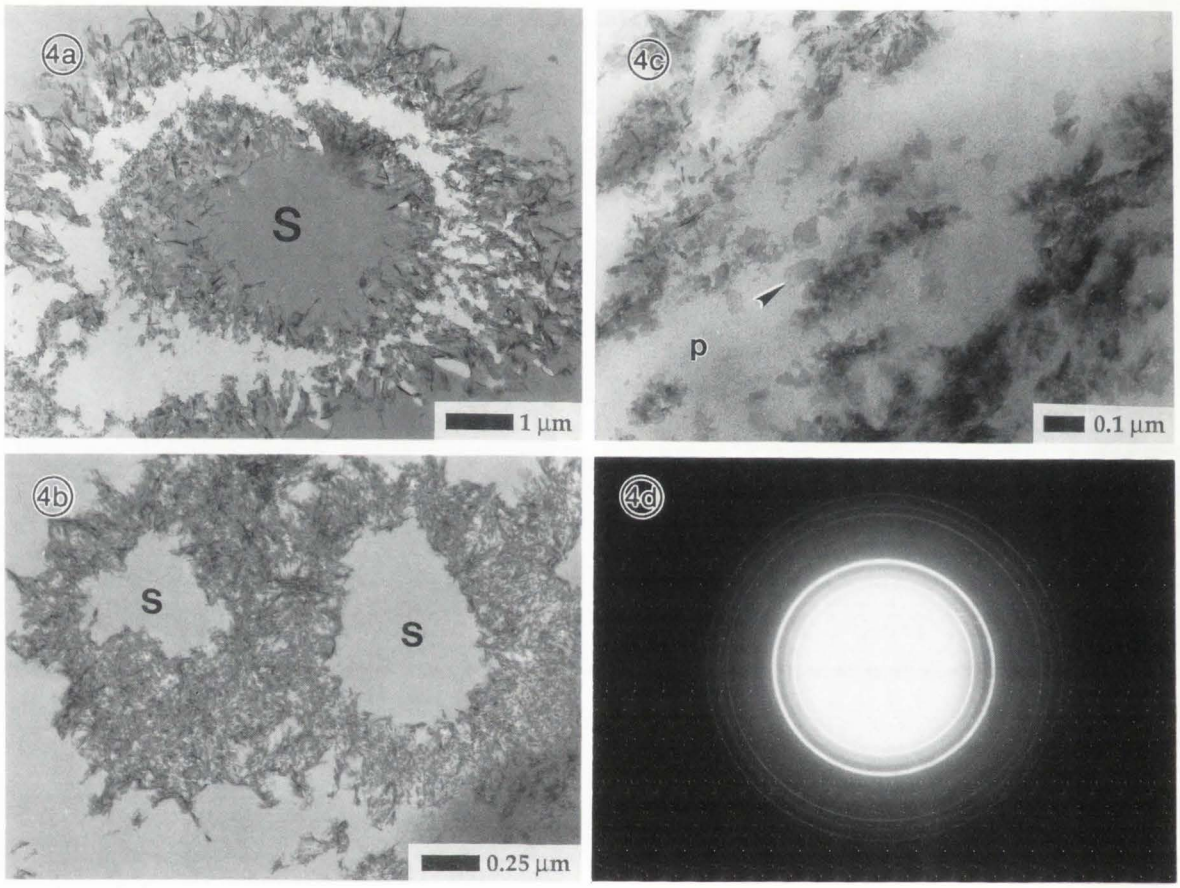

Figure 4: Ultrastructural Features of Mineralized Collagen Fibers Formed at Neutral and Alkaline pHs. The micrograph presented in figure 4a is of a section obtained perpendicular to the axis of the mineralized fiber through spherical structures (S) on the surface of the fiber. These structures appear as hollow aggregates of thin crystal plates. Similarly, the micrograph in figure 4b is of a perpendicular section of the mineralized fiber formed at pH 8.4, and shows cross sections of the crystal spheres (S) observed on the surface of the fiber. These structures are also hollow and composed of fine crystalline plates. Crystal formed in the interior of the collagen fiber (figure 4c) are characterized as thin roughly rectangular plates (P). Figure 4d is a typical selected area electron diffraction pattern obtained from mineral formed within the collagen fiber, and is characteristic of hydroxyapatite.

demineralized bone matrix [15] in incubating media containing calcium and phosphate at acidic pHs. Renal stones are composed of a variety of crystal phases including brushite crystal. In a study simulating the conditions of renal crystal growth, LeGeros and Morales [32] found brushite crystal formation in gel systems with similar morphological features to the brushite crystals in this study. A general feature common to both crystals formed at pH 4.65 and pH 5.15 was the presence of stellate aggregates of needle like crystals. This stellate pattern of "effloresecence" was also observed by Clarke et al. [15] for brushite crystals formed on hydroxyapatite and demineralized bone matrix when exposed to saturated solutions of hydroxyapatite powder in acidic water. The stellate aggregates observed by Clarke et al. [15] were limited to a maximum of 16 petals, found on the surface of a tricalcium phosphate carpet which formed preferentially on the bone matrix at low pH. Brushite crystals identified by FT-IR [14] were seen directly on the surface of the collagen fiber, with no morphological evidence of a intermediary carpet phase at acidic pH in our study. This exclusive formation of brushite is attributed to the differences in solution conditions (ie spontaneous precipitation of calcium and phosphate versus dissolution and reprecipitation of powdered hydroxyapatite), and the geometry and composition of the protein substrate. The morphological change of angular needles (pH 4.65) to cubic rods (pH 5.15) for the crystals formed on the reconstituted collagen fibers may be indicative of a mixed mineral phase at

the slightly higher pH [33]. However, this hypothesis can only be confirmed with more structure specific studies such as polycrystalline or single crystal x-ray diffraction of the mineral formed at each pH. In addition, the large flat plates formed at pH 4.65 were not seen at pH 5.15, and were replaced by large rectangular rods which showed an overall preferential orientation with the axis of the fiber. Whether or not these oriented crystal are the result of matrix mediated crystal growth cannot be determined by the micrographic analysis presented in this investigation.

At pH 7.0 the crystal was observed as a fairly uniform coating of small plate-like crystals, as well as regions containing spherical aggregates of these fine crystalline plates. This morphology corresponds to hydroxyapatite crystals developed on collagen [29] in saturated solutions of calcium and phosphate and on the surfaces of bioactive glasses incubated in physiological solutions [1, 28]. Mineralization at pH 8.4 exhibited less of the uniform carpeting, and the spherical structures were smaller and dispersed over the surface of the collagen fiber.

Transmission electron micrographs of the spheres formed at neutral and alkaline pHs obtained from sections oriented perpendicular to the fiber axis show the spheres as hollow structures. This "egg shell" morphology was also observed by Brown et al. [10], who proposed that this structure resulted from a reaction in which the product (hydroxyapatite) surrounds the reactant, which is slowly consumed as the shell is formed.

The shape and size (~ 95 X 53 X 5 nm) of the hydroxyapatite crystals formed within the collagen fibers at neutral and alkaline pHs are within the range of sizes reported for crystals isolated from a variety of mineralized tissue sources [40] Comparison of the crystal structure determined from selected area electron diffraction patterns with the structures of a variety of calcium phosphate minerals determined by X-ray diffraction indicate that these mineral crystals formed in the interior of reconstituted collagen fibers are hydroxyapatite. The selected area electron diffraction patterns lacked evidence of c-axial orientation, indicating that the bulk of the mineral formed in the reconstituted collagen fiber is randomly oriented.

Preliminary comparison of apatite crystals formed at pH 7.0 and pH 8.4 imply that the mineral formed at the more alkaline pH are smaller in both length and width. This preliminary observation follows data regarding amorphous calcium phosphate precipitation and recrystallization as a function of pH [7]. The authors conclude that at higher pH, a number of factors including; increased nucleation rate, and decreased crystal solubility at higher pH, result in the formation of more plentiful and smaller amorphous calcium phosphate particles, subsequently, smaller and more plentiful apatite crystals.

Prior studies of the mechanical properties [14] of the mineralized collagen fibers as a function of pH, indicate that with mineralization the tensile strength of the fiber increased over the acid - alkaline pH range, with respect to the unmineralized fiber. This result may imply that the mineral forms with some degree of association with the collagen, and the fibril substructure serves a role beyond a passive support for the crystals.

The results of the morphological and preliminary ultrastructural studies reported here, combined with the results of mechanical tests of the mineralized matrix and FT-IR analysis [14], indicate that the in vitro mineralization of reconstituted collagen fibers may provide a useful model system in the study of calcium/phosphate mineral deposition in a type I collagen scaffold in vitro. The reconstituted collagen fiber was an aligned fibrillar substructure analogous to the aligned collagen fibril scaffold in turkey tendon and may prove useful in the evaluation of physical and chemical interactions that arise in the absence of cells and other regulatory factors during mineralization. With further refinement and development, this system may allow the formation of biomaterials that mimic the structure of bone as previously reported [14]. Future studies are planned to investigate; transmission electron microscopic analysis of sections obtained parallel to the axis of the collagen fiber, the distribution of the mineral phase across the fiber, the effect of cross-link density on fiber mineralization, and the effects of cyclic axial stress on the reconstituted collagen fibers during mineral deposition.

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Discussion with Reviewers

Reviewer I: How consistent were the morphological features of the mineral at each of the pH values investigated?

Authors: As noted in the Materials and methods section, 15 fibers from 3 separate experiments at each pH point investigated were submitted to morphological evaluation via scanning electron microscopy. The micrographs presented in figures 2A-2D and 3A-3D characterize the morphological features of the mineral crystal formed at each pH point. These features were uniformly consistent in all samples examined within each set of mineralized fibers.

Reviewer I: What is the significance of the brushite crystals grown at acidic pH in relation to biological mineralization?

Authors: X-ray diffraction studies of mineralized region of the epiphyseal growth plate [5] have presented evidence for the existence of brushite mineral in association with apatite during the early stages of mineralized tissue development. However, these observations were not corroborated by recent and more sensitive electron spectroscopic diffraction of the early and mature stages of mineral formation in the epiphyseal growth plate [1]. Consequently, the significance of brushite crystal formation in mammalian mineralized tissue formation is a subject for debate. However, brushite crystals are known to represent a component of the mineral phases formed in renal calculi, in addition to hydroxyapatite, calcium oxalate and struvite [4]. The solution environment during kidney stone formation is often acidic and mineral formation occurs in the presence of proteins.

L. Addadi: There is an important piece of missing information relating to all the described experiments: the comparison between the crystals formed on the fibers and those formed in the environment of the dialysis bag. Is there anything different between them, that may indicate an active role of collagen of the collagen in the crystallization?

Authors: Samples of the mineral formed within the dialysis bag were filtered from the incubating solution at the conclusion of each experiment and immediately viewed under light microscopy. The crystals formed at each pH point were directly comparable to the morphological features observed under scanning electron microscopy. Specifically, at neutral and alkaline pH the mineral consisted of small flat crystals and aggregates of crystals, and at acidic pH the crystals were much larger geometric structures, though no stellate aggregates were noted for the crystal formed in solution at acidic pH. Thus no morphological difference can be noted between the crystal formed in the solution surrounding the fiber and the crystal formed on the surface of the fiber. However, more detailed structural investigations [2] of the pH dependent mineralization of the reconstituted collagen fibers clearly show distinct changes in the mechanical properties of the matrix subsequent to mineralization indicating that the reconstituted collagen serves an active role in the mineralization process.

L. Addadi: The reconstituted collagen fibers are soaked in alcohol and dried during the preparation. There is no mention of the effect that this treatment may have on the protein. Collagen is modified by this procedure, and this could influence its behaviour in crystallization.

Authors: Organic solvents including alcohol have been used extensively in the study of proteins [3], and are noted to alter the balance of electrostatic charges on the protein and change the organization of dipolar moments. However, these effects are also shown to be reversible [3]. In addition, ethanol fixation is a common technique used in the preparation tissue for immunohistochemistry [5], largely because any denaturing effects are mild and reversible. The deleterious effects of alcohol treatment are most pronounced in soluble proteins as opposed insoluble proteins such as the type I collagen employed in these investigations. Rougvie and Bear [6] noted a significant decrease in the axial D - spacing of collagen subsequent to dehydration, however, transmission electron micrographic measures of unfixed and embedded mineralized collagen fiber D - spacing indicated no change following dehydration [2]. It is believed that any changes in the charge characteristics of the collagen caused by dehydration are minor and reversible. Dr. Addadi raises a very good question, and based on our evidence at this point we feel the effects of the treatment methods, specifically the alcohol immersion and subsequent drying, are minimal. However it is a question worth pursuing, and will be incorporated in future studies.

See next page for "Additional References"

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