Synthesis of Calcium Phosphate Nanoparticles in Collagen Medium

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Summary: Mineralization of the proteic matrix composed mostly of collagen type I is controlled by specific interactions of ions Ca^{2+} and PO_4^{3-}, present in the biological fluid, with the matrix, and by the diffusion of these ions. The specific interactions and the diffusion of ions combined; result in the nucleation and formation of calcium phosphate particles. Moreover, they control the morphology, size, crystallinity and composition of the particles. In this work, precipitation of calcium phosphate particles in a collagen matrix type I was carried out through impregnation of the matrix with a Ca^{2+} and PO_4^{3-} solution, with Ca/P ratio 1.67 and pH 2.5. Precipitation of particles associated with matrix structuring was carried out by adsorption of gaseous ammonia.

Keywords: biomaterials; calcium phosphate; collagen; mineralization; nanoparticles

Introduction

The bone matrix that composes bone tissue may be considered a true composite, composed of interconnected calcium phosphate nanoparticles (c.a 70% m/m), originated in the mineralization of a protein matrix composed mostly of collagen type I.[1,2]

The interaction between ions present in the body fluid and the protein matrix leads to the formation of calcium phosphate nanocrystals, which is the mineralization of the protein matrix. These calcium phosphate nanocrystals, similar to carbonated hydroxyapatite in composition, form agglomerates and interconnected structures, like the ones observed in micrographs of inorganic bone phase.[3–5] The formation and morphology of the bone matrix, as well as the calcium phosphate nanocrystal aggregation are controlled by several parameters. Control of these processes has been attributed to the chelate effect of carboxylate groups present in collagen on ions Ca^{2+}, to ion diffusion in the protein matrix, as well as the supersaturation of ions Ca^{2+} and PO_4^{3-} in body fluid when compared to the solubility of carbonated hydroxyapatite at biological pH.

With a view to understanding *in vivo* mineralization, the *in vitro* mineralization of collagen gels and of polymeric gels containing carboxylate groups has been studied. In order to mimic *in vivo* mineralization conditions, it is imperative that the conditions that are assumed to result in the formation of bone matrix be used as a model.

In order to mimic the formation of a bone matrix, collagen or polymeric gels are generally impregnated with Ca^{2+} and PO_4^{3-} solutions, at pH values that do not result in supersaturation, compared to carbonated hydroxyapatite solubility. After the gel has been impregnated, pH is increased either by addition of a base[6,7] or by homogeneous precipitation, due to urea hydrolysis.[8,9]

In this work, precipitation of calcium phosphate particles in a collagen type I matrix was carried out through impregnation of the matrix with a Ca^{2+} and PO_4^{3-} solution, with Ca/P ratio 1.67 and pH 2.5. Precipitation of particles associated with

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matrix structuring was carried out by controlled adsorption of gaseous ammonia.

Impregnation of the matrix with a Ca^{2+} and PO_4^{3-} solution, followed by pH increase through controlled absorption of gaseous ammonia results in mineralization of the matrix at body temperature (37 °C) and promote the action of calcium phosphate nucleation sites, represented by carboxylate groups of collagen.

Experimental Part

Preparation of Collagen Matrix

Collagen matrix type I was obtained from tendons of Wistar rat tails, through dissolution of tendons in acetic acid 0.5M.[10]

The colloid was filtered in order to eliminate impurities and then a NaCl solution was added to it, so as to make the final saline concentration 0.7M. At this concentration, there is almost exclusive precipitation, or fibrilogenesis, of collagen type I. In order to finally isolate the collagen matrix, the mixture was centrifuged at 10 °C and 10.000 rpm.[10]

Mineralization of the Collagen Matrix

The collagen matrix previously isolated was dialyzed at 4 °C for 36 hours in deionized water in order to eliminate residual NaCl.[10] During dialysis, volume of the dialysis bag and collagen mass were adjusted, so that at the end of dialysis, a collagen gel at concentration 0.1g/mL would be obtained. After dialysis was over, the bag containing the collagen gel was soaked in a solution 0.167M Ca^{2+} and 0.1M PO_4^{3-} (Ca/P = 1.67), at pH 2.5. The ionic solution was changed every 12h, three times.

Mineralization of the collagen matrix after impregnation with Ca^{2+} and PO_4^{3-} solution was carried out through controlled gaseous ammonia permeation of the gel.

Deproteination of Mineralized Collagen Matrix.[11]

The mineralized matrix was treated with 1mL of hydrazine hydrate (60%) for 12h, at 27 °C, so as to eliminate the protein matrix and therefore allow determination of the morphology of calcium phosphate particles and aggregates formed during mineralization. Alternatively, the mineralized matrix was also treated with 1mL each day of hydrazyne for 72 h at 60 °C. After deproteination, the material was washed in ethanol, to which drops of NH_4OH had been added to keep the medium basic. This procedure was repeated 3 times and after each wash the solid was isolated by centrifugation.

To draw a comparison with calcium phosphates resulting from mineralization of collagen matrix, femur bones of Wistar rats at 1 year of age were also deproteinated with hydrazine, for 72h at 60 °C.[12]

Techniques for Material Characterization

The collagen matrices (both the mineralized and the not mineralized), the calcium phosphates isolated from the mineralized matrices and the inorganic bone phase were characterized by XRD, SEM, FTIR and ICP-OES.

Results and Discussion

Interaction Between Calcium Ions and Collagen

The interaction between calcium ions and the collagen matrix was evaluated by IR spectroscopy of the material obtained after it had been soaked in the Ca^{2+} and PO_4^{3-} ionic solution, at pH 2.5. Figure 1 presents the FTIR spectra obtained with the ATR

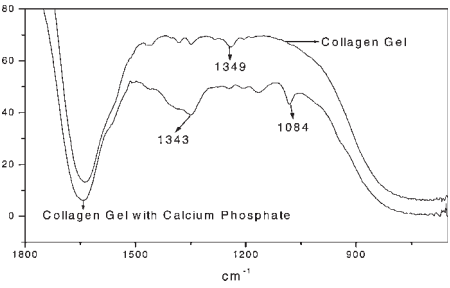

Figure 1.
Infrared Spectrum obtained by ATR of pure collagen gels and collagen gels with Ca^{2+} and PO_4^{3-} (FTIR).

technique, both for collagen gels and for collagen gels soaked in Ca^{2+} and PO_4^{3-}.

The FTIR spectra (Figure 1) of collagen gels and collagen gels soaked in Ca^{2+} and PO_4^{3-} are very similar up to 1500 cm^{-1}. However, the peak at 1349 cm^{-1}, corresponding to the stretching of groups COO– in pure collagen, shifts to 1343 cm^{-1} in the collagen soaked in Ca^{2+} and PO_4^{3-} solution.

The shift observed in the infrared spectrum might be attributed to a possible interaction between calcium ions and carboxylate ions of collagen, as proposed by Rhee et al.[13] The interaction between calcium ions and anionic groups of collagen, chiefly with the carboxylate groups, has been considered pivotal for the mineralization of the collagen matrix, playing an important role in the formation and arrangement of calcium phosphate particles.[13,14]

Composition of the Material Precipitated in Collagen Matrix

Figure 2 presents the FTIR spectra of the deproteinated materials: the collagen matrix after mineralization, as well as the inorganic bone phase of Wistar rats. The IR spectra of the deproteinated mineralized collagen and of the inorganic bone phase are similar. The peaks observed in Figure 2 might be attributed to the ν_3 antisymmetric stretching of PO_4^{3-} or ν_6 stretching of PO_3 in HPO_4^{2-} (1100, 1093 and 1047 cm^{-1}) and ν_4 bending of PO_4^{3-} or ν_4 bending in HPO_4^{2-} (616 and 581 cm^{-1}).[15–17] The similarity observed between the spectra indicates that both calcium phosphates, the one formed within the collagen matrix and the inorganic bone phase, have very similar composition.

The deproteinated mineralized collagen and the inorganic bone phase were dissolved in nitric acid and their Ca/P ratio was determined by ICP-OES. Table 1 shows the results of these determinations.

Effect of Collagen Matrix Upon Morphology of the Precipitate

The effect of the collagen matrix upon the morphology of calcium phosphate nanoparticles obtained in the mineralization process was evaluated by scanning electron microscopy. Figure 3 and 4 show the results of hydrazine-deproteination for 12 hours at 27 °C, and for 72 hours at 60 °C, respectively.

In Figure 3 it is possible to see globular agglomerates of calcium phosphate nanoparticles coated by the collagen matrix, what indicates that they were formed within the collagen matrix. Figure 3B, which represents a magnification of micrograph 3A, makes particle size (approximately 0,5 μm) readily visible.

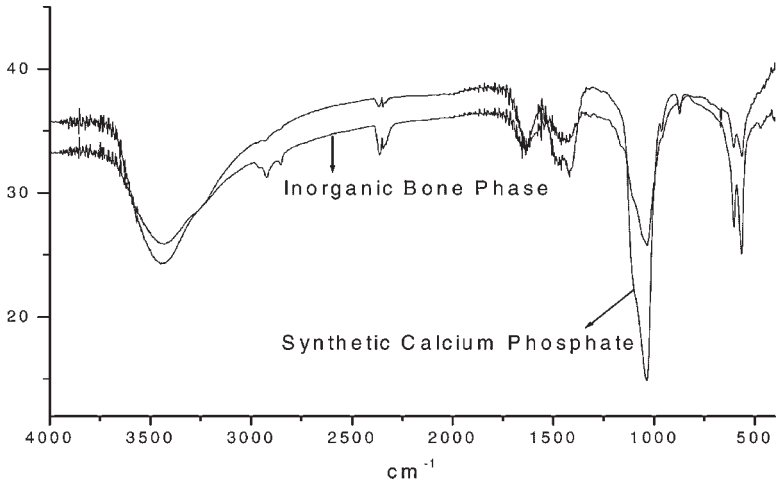

Figure 2.
FTIV spectra of the inorganic bone phase and deproteinated mineralized collagen.

Table 1.
Ca/P ratio obtained by ICP-OES.

	Deproteinated mineralized collagen	Inorganic bone phase
Ca/P ratio	1.67 ± 0.05	1.71 ± 0.05

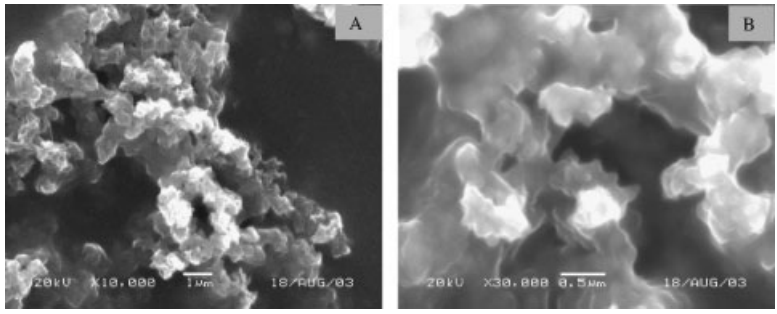

Figure 3.
Micrographs of deproteinated mineralized collagen and of calcium phosphate prepared after treatment with hydrazine for 12 hours at 27 °C. Figure 3B, represents a magnification of micrograph 3A.

The micrograph in Figure 4, obtained after the action of hydrazine for 72 hours at 60 °C, shows that the particles observed in Figure 3 are actually formed from calcium phosphate nanoparticle agglomerates, encased in the globular structures observed in Figure 4.

Particle Size Determination

The materials precipitated on the collagen matrix and the inorganic bone phase were analyzed by X-ray diffraction for determination of crystallite size, using Debye Sherrer equation.[18] Figure 5 presents the diffractograms obtained for the calcium phosphates precipitates both on the collagen matrix and on the inorganic bone phase of Wistar rats. Although broad, the peaks at 26° (002) and 32° (211), present diffraction patterns typical of apatites, possibly hydroxyapatites.[19,20] The resemblance between the diffractograms of the two distinct materials indicates a similarity in composition between them.[21]

Application of Debye-Scherrer equation to peak width at 25.8° 2θ (plane 002) results in a mean crystallite size of 20 nm, for the calcium phosphate precipitated on the collagen matrix and for the inorganic bone phase (Table 2), what demonstrates that the globular agglomerates observed in Figure 3 and 4 are actually formed from yet smaller particles.

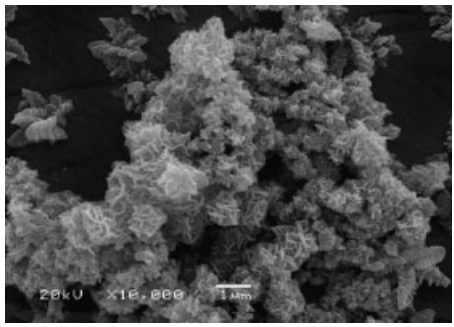

Figure 4.
Micrograph of acicular agglomerate materials, obtained by the action of hydrazine upon the composite collagen/ calcium phosphate for 72h at 60 °C.

Conclusions

The effect of the collagen matrix on the mineralization process, due to calcium phosphate nucleation sites, which are represented by carboxylate groups of collagen, results in the formation of globulars agglomerates of calcium phosphate nanoparticles. and X-ray diffractometry analyses

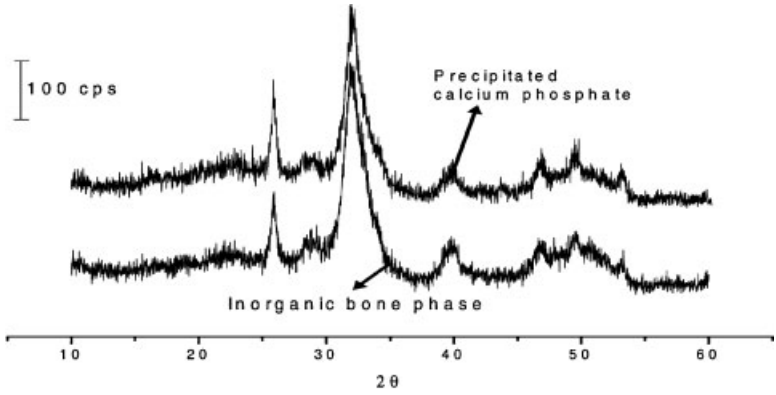

Figure 5.
X-ray diffractograms of calcium phosphate precipitated on collagen and inorganic bone phase.

Table 2.
Crystallite size of synthetic calcium phosphates and inorganic bone phase, obtained by Debye-Scherrer.[18]

Material	Size (nm)
Synthetic Calcium Phosphate	20 nm
Inorganic Bone Phase	20 nm

show that calcium phosphate nanoparticles present reduced crystallinity and mean crystallite size of 20nm. The crystallinity and mean crystallite size are similar to those of the inorganic bone phase.

Determination of calcium phosphate nanoparticle composition results in Ca/P ratio of 1.67, which is the same as that of hydroxyapatite.

The mineralized collagen matrix might have potential for application as biomaterial.

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