

ON THE COLLAGEN MINERALIZATION. A REVIEW

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Collagen mineralization (CM) is a challenging process that has received a lot of attention in the past years. Among the reasons for this interest, the key role is the importance of collagen and hydroxyapatite in natural bone, as major constituents. Different protocols of mineralization have been developed, specially using simulated body fluid (SBF) and many methods have been used to characterize the systems obtained, starting with methods of determining the mineral content (XRD, FTIR, Raman, High-Resolution Spectral Ultrasound Imaging), continuing with imaging methods (AFM, TEM, SEM, Fluorescence Microscopy), thermal analysis (DSC and TGA), evaluation of the mechanical and biological properties, including statistical methods and molecular modeling. In spite of the great number of studies regarding collagen mineralization, its mechanism, both *in vivo* and *in vitro*, is not completely understood. Some of the methods used *in vitro* and investigation methods are reviewed here.

Keywords: bone, collagen, hydroxyapatite, mineralization, scaffold, biomaterial.

Introduction

Research on *in vitro* mineralization of self assembled collagen fibrils is a largely approached dynamic domain, since there is great interest in bone grafts based on this kind of composite materials.

The name bone designates a family of natural materials, nanocomposites presenting a multi-level complex hierarchical structure based on mineralized collagen fibrils [1-2].

Collagen is a complex protein having a repetitive sequence of amino-acids, in particular: glycine, proline and hydroxyproline (Fig. 1). More than 20 types of collagen have been identified until now, but collagen type I and collagen III are the most abundant in nature [3-6]. Collagen can be found in different parts of the human body, such as: cornea, skin, tendon, cartilage, and bone [7]. Collagen type I is the most abundant protein in the natural bone [8-9].

Bone is one of the most perfect natural structures

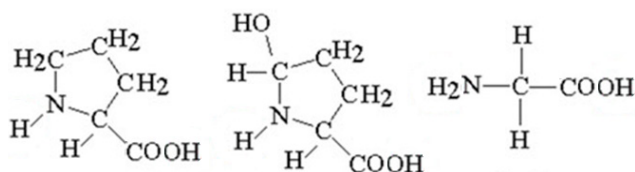


Figure 1. Chemical structures of the collagen main constituents: proline, hydroxyproline and glycine.

[10] and its reconstruction has great importance in treating different orthopedic diseases including cancer. Bone is the main structure to support the body and protect internal organs in vertebrates [1,11], and also it is important in maintaining the concentration of inorganic ions (Ca^{2+} , PO_4^{3-}) through continuous resorption and remodeling [2,12].

In bone, mineralized collagen usually contains calcium phosphate based crystals, having as principal components Ca^{2+} and PO_4^{3-} (phosphate) ions, but also small amounts of other cations, such as Mg^{2+} , and anions: CO_3^{2-} (carbonate), OH^- (hydroxyl), Cl^- (chloride), F^- (fluoride), citrate and other [13]. These calcium phosphate phases

implied in the mineralization of collagen fibrils are similar to hydroxyapatite (HAP) [14].

Bone contains, in principal, 3 types of components, namely: inorganic materials (65-70%, especially hydroxyapatite, HAP, which confers hardness), organic mass (20-25%): collagen type I, predominantly, which offers the elasticity necessary for movement and small quantities of osteonectin and osteoclastin that lead to the regeneration of the bone), and water associated with collagen (10%) [12,15].

But composition alone does not account for the outstanding mechanical properties (strength and toughness) of bone, which are essentially determined by its nanostructure, organized on seven levels of hierarchy [1,16-17]. In all bone materials, there is the same basic building block: a collagen fibril, with HAP crystals (platelets), with their c-axes [0 0 1] aligned preferentially parallel to long axis of the fibril. These fibrils were beforehand arranged by self assembly in a matrix presenting a periodic array of hole and overlap zones [17]. HAP crystals probably nucleate in the hole zones, but outgrow them and are stored between tropo-collagen molecules (triple helices of collagen peptides), thus generating an interpenetrating organic-inorganic nanocomposite [18]. Due to the metastability of the extremely small HAP nanocrystals, they can be resorbed by osteoclasts during the natural remodeling processes of the bone [19]. Afterwards these mineralized fibrils self-assemble further into higher levels of structure, for instance in parallel arrays that rotate across the concentric lamellae of osteons [1,20] with further hierarchy directed by osteoblasts as they lay down a trabecular and cortical bone macrostructure [12]. As a particular case, mature enamel - the hardest tissue in the body - contains 95% apatitic mineral, 4% water and less than 1% of organic matrix [16].

In the natural bone, mineralization of collagen is the result of complex biological processes {see, for example [7,21-22]} in which deposition of inorganic salts is induced by a template of collagen network formed through self-assembly [23]. It was intensively studied over the last 50 years, but the exact molecular mechanism of mineralization of bone collagen is not completely elucidated till now [23-24]. Since type I collagen by itself cannot induce apatite nucleation [25], non-collagenous proteins such as osteonectin and osteocalcin [26] contribute to the stabilization of amorphous calcium phosphate phases as nanoprecursors (sequestration motif) [27-28], and to the initiation of nucleation and hierarchical assembly of apatite within the collagen scaffold (templating motif) [29]. The present review will present only *in vitro* methods for collagen mineralization.

Different ways of preparing mineralized collagen

At present there is an increasing demand for improved bone graft substitutes in order to mimic the

properties of natural bone, a great number of organic/inorganic composites have been proposed.

Three-dimensional scaffolds are used in tissue engineering for cell cultures, to form a mature matrix for implantation into the body [30]. The scaffold is the initial support for the cells, and it has an important effect on cell processes (proliferation, migration, etc) [31]. It must have a series of characteristics such as: high porosity, mechanical stability, biocompatibility and biodegradability [15]. Mostly collagen and hydroxyapatite have been considered as scaffold materials for bone tissue culture for regeneration.

As shown above, fibrous type I collagen is chemically and structurally similar to natural extracellular matrices [33], and it is combined in bone with (carbonated) hydroxyapatite in a hierarchically organized biocomposite tissue, from nano to macroscopic scale [1]. It is commonly used as a biomaterial in scaffolds, due to its stability, bioactivity and biocompatibility. Collagen gels support cellular proliferation and osteogenic differentiation in a three-dimensional matrix [34]. Bovine tissues are the main commercial source of collagen, but also other sources were considered, especially after Bovine Spongiform Encephalopathy became a major concern. For instance, collagen-rich fish solid waste was taken in consideration as an alternative source [35].

Simple collagen scaffolds and constructs could be used in bone implants without mineralization, but they would need more than 2-3 weeks for osteointegration [36]. This is why as a rule composites of collagen and HAP are used [37], but also other inorganic materials were considered, such as silica. The mineralization process improves the mechanical resistance of collagen and makes it a suitable matrix for bone repair [38].

Such mineralized matrices are a valuable biomedical tool for regulating diverse phenotypic activities of stem and progenitor cells in the repair and regeneration of bone tissues [39-40], but are also useful in the study of bone diseases, e.g. osteoporosis or cancer metastasis [41].

Early bone grafts used to be based on highly crystalline sintered HAP, but this material is not readily resorbed by the body, so now research is focused on composites based on amorphous HAP [10].

Among the methods of preparing mineralized collagen, we can distinguish [12,15]:

- the direct blending of collagen and mineral crystals, i.e. simply mixing together collagen and mineral nanoparticles;
- the co-precipitation of mineral during collagen fibrillogenesis;
- the “biomimetic” approach of immersion of collagen scaffolds in simulated body fluid, SBF [7] to coat the collagen scaffold surfaces with minerals.

SBF is also used for the direct surface modification of metallic and other implants The classical Kokubo

solution formulated with ion concentrations similar to blood plasma [42] has the following ionic composition (mmol/L): Na^+ 142.0; K^+ 5.0; Mg^{2+} 1.5; Ca^{2+} 2.5; Cl^- 147.8; HCO_3^- 4.2; HPO_4^{2-} 1.0; SO_4^{2-} 0.5. As a precursor for the collagen mineralization, it is prepared by mixing in ultrapure water the following solutions: NaCl , NaHCO_3 , KCl , $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, CaCl_2 , and Na_2SO_4 , adjusted to physiological pH (7.4) [23].

The enrichment of SBF with transition metal ions was also proposed [23]. Ions of this kind (Co^{2+} , Ni^{2+} , Zn^{2+} , Fe^{3+} , Mn^{2+} , Cu^{2+}) used to dope HAP, play an important role as trace elements for the growth and metabolism of bone tissues, in preventing and treating bone diseases.

The co-precipitation method to obtain collagen/calcium phosphate composites implies the self assembly of collagen fibrils from soluble molecules and the precipitation of calcium phosphate (mostly HAP) in aqueous solution [43-45]. For instance, a basic solution containing Ca^{2+} was added drop-wise to a solution containing dissolved type I collagen in phosphoric acid. The self assembly of collagen takes place simultaneously with the precipitation of HAP at pH 9-10, giving their nanocomposite [44,46]. Instead of collagen, gelatin – its denaturated form – was also used [47]. Such materials present a rather good bioactivity, but have poor mechanical properties, which limit their applications [48].

The most commonly used surface mineralization techniques [10] include soaking the scaffold in (SBF) [49-50] and the alternating dipping method [51], the last being faster (hours instead of days duration). But it is difficult for the solution to reach the interior of the nanofibrous block. An *in situ* diffusion method [52] tries to obtain collagen/nanoHAP scaffolds with a concentration gradient, from solutions containing Ca^{2+} and PO_4^{3-} ions, which precipitate HAP crystallites on the collagen fibrils. In order to control the ionic diffusion during the mineralization process, a dual membrane diffusion system was proposed, with a cation-selective membrane and an anion-selective dialysis membrane and a carboxymethylated collagen fibril substrate [53].

Mineralization of scaffolds induced by incubating collagen matrices in SBF or by activation of calcium and phosphate secretion from cells loaded within the matrix [54] improves the bonding of the implants to the bone and the osteogenic differentiation of bone-forming stem and progenitor cells [40]. But such collagen gels encapsulating calcium-secreting cells lack rigidity and tend to shrink significantly. [40, 55].

Such conventional *in vitro* collagen mineralization methods, realized by the nucleation and growth technique, using a direct combination of reactant ions and/or various forms of simulated body fluid as the reaction media [56-60] cannot reproduce the native nanostructure of bone which arises from intrafibrillar mineralization. HAP crystals appear randomly oriented in spheritic clusters on the surface

of the collagen scaffolds [17]. It is possible that the external mineral crust formed prevents further mineralization of the collagen fibrils beneath the surface [17,61].

Since these collagen-calcium phosphate composites have inadequate mechanical properties, in order to improve these characteristics synthetic polymers or agents for crosslinking are added. Among the polymers used to strengthen the mineralized collagen we can cite poly (lactic acid (PLA) [62] or the copolymer poly(lactic-co-glycolic acid) (PLGA) [40]. Some cross-linking agents used are glutaraldehyde [63-64], succinic anhydride, and acyl azide [65], 3-Aminopropyl triethoxysilane [36]. A disadvantage of these agents is their cytotoxic properties.

Of course, there is also the possibility to apply collagen/ calcium phosphate composite coatings on the surface of metallic implants; so we obtain a bioactive surface together with the high mechanical characteristics of the metal core [66]. To this aim, the use of electrolysis proved to be useful for collagen self-assembly and calcium phosphate mineralization [48]. Electrochemical processes are also implied in the electro-deposition of hydroxyapatite on collagen [67], or in the electrochemically assisted deposition of coatings from a $\text{Ca}^{2+}/\text{H}_x\text{PO}_4^{(3-x)}$ electrolyte at physiological pH and temperature [68].

The methods using SBF are often named “biomimetic”, but as a matter of fact, they do not accurately mimic the *in vivo* bone formation process, particularly intrafibrillar mineralization, and often result in scaffolds that are not suitable for bone tissue engineering.

A fundamental breakthrough in mimicking intrafibrillar mineralization was reached by the method of the polymer-induced liquid precursor (PILP) process [69-70]. The addition of acidic polypeptides to the mineralization solution induces or stabilizes an amorphous highly hydrated precursor to the mineral (HAP), with a liquid-like character. This process could even explain the biological biomineral morphogenesis in vertebrates and invertebrates [71]. By this process, intrafibrillar mineralization of collagen can be realized *in vitro*: the HAP nanocrystals are embedded and [0 0 1] aligned within collagen fibrils, as in the nanostructure of natural bone [71-73]. The acidic polypeptides mimic the role of the noncollagenous proteins such as osteonectin and osteocalcin in the natural process, and permit intrafibrillar mineralization [71,74-77].

The mineralization by the PILP process of individual collagen fibrils were studied and the products were characterized [71,75,77]. The randomly oriented bundles of fibrils in the form of porous collagen scaffolds were also investigated [17,71,77,78-79].

In one of the approaches, polyaspartate was used to mimic the acidic non-collagenous proteins involved in bone formation, and alkaline phosphatase to provide a slow release of inorganic phosphate ions from a phosphate ester [17]. Polyaspartic acid, together with K_2HPO_4 and CaCl_2 were used in a perfusion-flow (dynamic) mineralization

technique, to obtain a collagen-HAP composite with structure and compositions similar to human trabecular bone [15]. Recently, osteopontin was used in such a process, and was shown to modulate both the mineralization reaction, and the cellular activity [80]. Polyacrylic acid was also used as sequestration analogue (to stabilize amorphous calcium phosphate into nanoprecursors) along with poly vinyl phosphoric acid (as substitute for matrix phosphoproteins), as templating analogue (to direct the nucleation and growth of apatite within collagen fibrils) for the remineralization in SBF of dentine collagen [21]. Small inorganic poly-phosphates (sodium trimetaphosphate and sodium tripolyphosphate) could also be used as templating analogues [22]. The role of phosphoproteins in the mineralization process was discussed [81].

Besides calcium phosphates, silica was used as an alternative in the mineralization of collagen, both because of the mechanical properties of the resulted innovative biomaterial, and for the stimulating effect of silicic acid in osteogenesis [82]. Composite from collagen and bioglass particles were prepared [33,83-84]. The same mineralization methods were used as for phosphates: silicification of previously assembled collagen fibrils [85], or simultaneous collagen fibrillogenesis and silica polymerization [86]. A silica/collagen/hydroxyapatite composite biomaterial was also obtained [14,87]. The modern method of intrafibrillar mineralization used for calcium phosphates was extended to biosilification [38].

Characterization of the mineralized collagen

The principal methods used to characterize the mineralized collagen are summarized in Table I, along with a few references to their application. All of them can offer important views on the collagen behavior under the

mineralization process and further perspectives on the mechanism involved.

The characterization of the mineralized collagen can be accomplished by using various techniques, most of the studies are using microscopy (SEM, TEM, AFM and fluorescence) to visualize the mineralized collagen fiber or spectroscopy (X-ray, FTIR, Raman, High-Resolution Spectral Ultrasound Imaging) to determine the chemical content of the samples and *in vitro* tests on cell cultures to see the interaction of the new material with the biological medium, but some other methods such as: determining the mechanical properties of the probes, analyzing the thermal behavior (DSC, TGA), or the absorption capacity and some molecular simulations of the interactions that may occur between collagen and the other components (specially hydroxyapatite) are mentioned.

Conclusions

Mineralizing the collagen is not an easy task; it can be accomplished under specific conditions and it depends on many parameters involved. A glimpse on the methods used to this aim evidences a continuous trend toward the development of biomimetic methods, in order to approach *in vitro* the natural processes of osteogenesis and to obtain materials presenting characteristics near to the ideal of natural bone. The degree of success can be estimated using a large variety of up to date investigation techniques and data processing methods.

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Table I. Methods of characterization for mineralized collagen.

Method	Reference
Morphological characterization:	
Scanning Electron Microscopy (SEM)	[12,15,23,33,36,40,48,53,68,78,80,88-96]
Transmission Electron Microscopy (TEM)	[12,16,21-22,24,48,53,78,89,95,97-100]
Atomic Force Microscopy (AFM)	[68,96,98]
Fluorescence Microscopy	[33,53,101]
Mineral content:	
X-ray Diffraction (XRD)	[12,15,23,33,36,40,74,80,88-90,93,95,102-103]
Fourier transform infrared spectroscopy (FTIR)	[21-23,33,36,48,68,87,89,91,94-95,97,102,105]
Raman Spectroscopy	[33,74]
High-Resolution Spectral Ultrasound Imaging	[55]
Mechanical properties:	
Modulus of elasticity	[15,33]
Biocompatibility:	
Cell culture	[15,33,35,50,80,95,97,100,102,106-116]
Thermal analysis:	
Differential Scanning Calorimetry (DSC)	[36]
Thermogravimetric Analysis (TGA)	[12,80]
Adsorption isotherms:	[21]
Molecular modeling:	[117,118]

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