Colagen / Hydroxyapatite Interactions in Composite Biomaterials

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The aim of this study is to investigate the interaction between pure collagen and mineral phases like hydroxyapatite. For that purpose, collagen fibers were mineralized starting from hydroxyapatite aqueous precursors. The mineral phase identification was made by XRD, the collagen-hydroxyapatite interaction was studied by IR spectroscopy, the microstructure of the composite materials was investigated by SEM and the amount of mineral phases' deposition was evaluated by ATD-TG. Ultrasonication was used in order to evaluate the strength of the collagen-hydroxyapatite bond. The obtained results showed that it was obtained a collagen – hydroxyapatite composite material, characterized by a strong interaction between the collagen fibers and the hydroxyapatite crystals, which can be successfully used as a bone substitute.

Keywords: collagen fibres mineralization, hydroxyapatite, electrostatic interactions

The in vivo synthesis of bone is not fully elucidated. Many research groups tried to obtain biomaterials with compositional and morphological similarity with the natural bone [1]. The biomimetic syntheses of bone substitute biomaterials imply many processes; the most important of them being collagen orientation and mineralization. There are many hypotheses about collagen mineralization process, some of them being contradictory [2].

The annual necessary of human bone grafts is in a continuous grow, due to an increasing number of fractures, congenital and non-congenital diseases, related also to the increased life duration of the population.

The history of bone grafting is starting in 1913, when Dr. D.E. Robertson tested a piece of cat's bone and a piece of human bone for grafting into dogs [3]. The microscopic analysis of implanted grafts showed after 20 days that the space between the grafts and the living bone is filled with new cancellous bone. These experiments are representing the premises for bone grafts development.

Due to the high increase of the necessity of bone grafting, autografts and allografts are not enough. To compensate this gap, artificial (synthetic) grafts are widely used [4]. The use of synthetic grafts exhibits also some advantages versus allografts, autografts and xenografts: possibility to obtain unlimited number / quantity of synthetic grafts, increased safety of use, without disease transmission risk, pain limitation by elimination of some secondary surgical interventions.

The natural bone contains mainly collagen and hydroxyapatite. This is the reason why many researchers are trying to understand and obtain hydroxyapatite/ collagen nanocomposites for hard tissue repairing. The composition of bone is influenced by many factors, such as: species, sex, age, type a.s.o.. The composition of bone [2] is presented in the table 1.

The *in vivo* bone biosynthesis is controlled by many factors, like: BMP (bone morphogenetic proteins) [5], transforming growth factors [6], cytokines [7], hormones [8-10], transcription factors [11], adhesion molecules [13] etc.

Many synthesis methods are used for the elaboration of composites with the same composition and structure with the natural bone. We are mentioning a few: *in vitro* collagen mineralization [14], thermally – triggered assembly of hydroxyapatite/collagen gels [15], vacuum infiltration of collagen into a ceramic matrix [16], enzymatic mineralization of collagen sheets [17], freeze drying and supercritical point drying [18].

One of the most promising bone graft material seems to be the collagen / nanohydroxyapatite composite, because of its very good compositional [2, 19] and structural similarity with the natural bone.

The role of each bone component is not very well known. It is generally accepted that the mineral phase, containing mainly hydroxyapatite, provides toughness and rigidity, while the organic matrix provides tensile strength and flexibility to the bone. In the composite materials, collagen and hydroxyapatite play the same roles [2] as in bone.

The mineral phase is deposited on the organic phase through electrostatic interactions between collagen's carboxyl groups and Ca^{2+} from hydroxyapatite.

Many researchers assert that hydroxyapatite is deposited on collagen only through non-collagenous proteins and citrate attached to it [20], while others say that mineralization occurs also on the pure collagen [21, 22].

In order to obtain good bone substitutes, we should understand the biosynthesis of natural bone and to control some parameters such as composition, hydroxyapatite crystals shape and size, collagen fibrils and hydroxyapatite crystal orientation.

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Table 1
BONE COMPOSITION [2]

Compound	wt %
Mineral phase	
Hydroxyapatite	60–66
Carbonate (mostly as carbonated hydroxyapatite)	4–6
Citrate	0.9–1.1
Na^+	0.35–0.7
Mg^{2+}	0.4–0.5
Others	Trace
Organic phase	
Collagen	20–25
Non-collagenous proteins: (osteocalcin, osteonectin, osteopontin, sialoprotein, BMP)	2 – 3
Others: (polysaccharides, lipids, cytokines)	Traces
Water	**8 – 9**

The orientation of collagen fibers and hydroxyapatite crystals in the composite is induced by the mutual interaction between collagen and hydroxyapatite in aqueous solutions. For better orientation, it is possible to use electric and / or magnetic field. The literatures data [23, 24] report the preparation of unidirectional oriented hydroxyapatite/ collagen composite using 10 T magnetic field, starting from calcium containing collagen solution and phosphate solution, at 37^0C. If a magnetic field is imposed perpendicularly to the rotation axis of the sample, unidirectional oriented collagen fibrils and hydroxyapatite crystals shall be obtained, due to their magnetic susceptibility anisotropy.

Experimental part

Insoluble collagen fibres (fig.1) were obtained in the Collagen Department of the Romanian National Research & Development Institute for Textiles and Leather, from calf hide, through a special chemical-enzymatic process. The resulted fibres were cross-linked with glutaraldehyde and dried through lyophilization process.

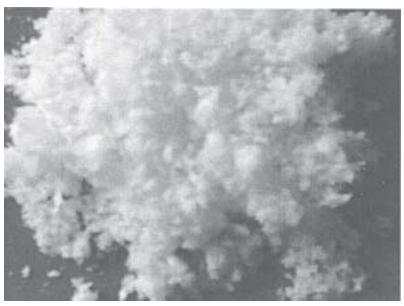

Fig. 1. Insoluble collagen fibres

The most important characteristics of collagen fibres are summarized in table 2, as stated by the provider.

The hydroxyapatite was obtained through a mineralization process, directly on the collagen fibres, starting from solutions containing Ca^{2+} and phosphate ions as precursors.

Table 2
INSOLUBLE COLLAGEN FIBRES CHARACTERISTICS

Collagen fibres characteristics	Value, %
Humidity	15.0
Total nitrogen*	17.5
Proteins*	98.0
Ash*	2.0
Glutaraldehyde*	1.0

* reported to dried material

The mineralization process was performed as follows: 1 g of collagen fibres was left for nucleation with 2.36 g $Ca(OH)_2$, dispersed in 100 mL distilled water, for 20 h, at 35^0C and *p*H = 9 – 10. After Ca^{2+} ions deposition on the collagen fibres, 6.80 g Na_2HPO_4 in 50 mL water was added, in small portions. The *p*H was fixed at about 9 – 10 by adding NaOH solution and the reaction mixture was left another 20 h, for hydroxyapatite precipitation on collagen fibres. Then the composite was dried in air, at 35 – 37 ^{0}C.

The obtained material was investigated through thermal methods, X-ray diffraction, IR spectroscopy and scanning electron microscopy.

X-ray diffraction analysis was performed using a Shimadzu XRD 6000 diffractometer at room temperature. In all the cases, Cu Kα radiation from a Cu X-ray tube (run at 15mA and 30 kV) was used. The samples were scanned in the Bragg angle, 2θ range of 10 – 80, with a sampling interval of 0.02.

SEM analyses were performed on a HITACHI S2600N electron microscope with EDAX, at 15 keV, in primary electrons fascicle, on samples covered with a thin silver layer.

For IR spectroscopy (Brucker) measurements, the spectra were recorded in the wave number range of 500 – 4000 cm^{-1}, with a resolution of 2 cm^{-1}, for insoluble collagen fibres and for collagen/hydroxyapatite composites.

The differential thermal analysis (DTA) coupled with thermo gravimetric analysis (TGA) was performed with a Shimadzu DTG-TA-50H, at a scan rate of 10 ^{0}C/min, in air.

Results and discussion

The aim of this study is to investigate the interaction between pure collagen and mineral phases like hydroxyapatite.

X-Ray diffraction

The XRD spectrum (fig. 2) proves the formation of hydroxyapatite (2Theta 25.92; 31.76; 32.3; 39.6; 47.2; 49.68; 53.26) on collagen fibres as the main mineral phase. Additionally, NaCl (2Theta 31.76; 45.46, 56.54) can also be detected, but the amount of NaCl can be neglected. The low crystallinity of the mineral phase is due to the *"nano scale"* of hydroxyapatite crystals. The XRD spectrum obtained is in good agreement with the XRD pattern of compact bovine bone powder [26].

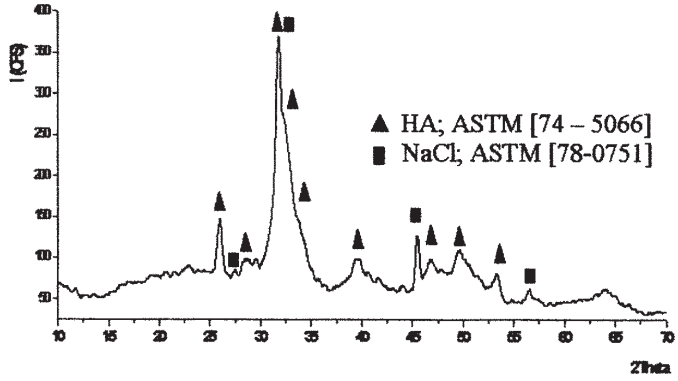

Fig.2. XRD patterns of collagen/ hydroxyapatite composite, obtained through the mineralization method direct on the collagen fibres

Scanning electron microscopy

The SEM analysis of mineralized collagen fibres give also information about the size of hydroxyapatite crystals, being in the nanometric range; the hydroxyapatite agglomerates size being of about 200 – 500 nm.

The microscopy images of collagen/ hydroxyapatite composites, presented in figure 3, show an uniform distribution of hydroxyapatite crystals on collagen fibres, as a result of the uniform distributions of the nucleation centers on the collagen fibres and the high ions mobility in solution.

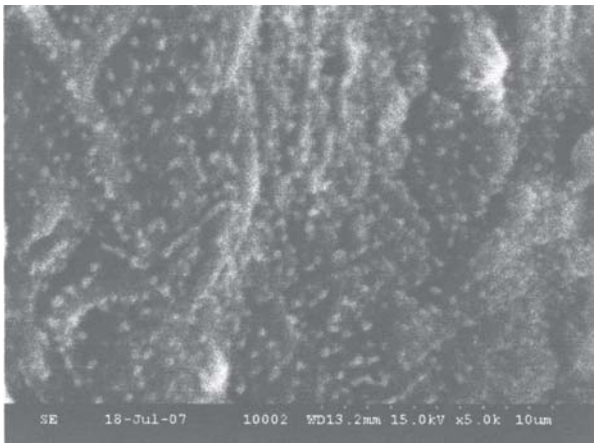

Fig. 3. The SEM micrograph of mineralized collagen fibres

Infrared spectroscopy

The IR spectra recorded for the collagen/ hydroxyapatite composite presents shifts for the carboxylate group peaks (1639.76, 1801.22 cm^{-1}), versus pure collagen peaks (1631.31, 1802.98 cm^{-1}), due to the electrostatic interaction between carboxylate groups and hydroxyapatite (fig. 4). Also, the hydroxyapatite deposition was confirmed; the peaks 1028, 563 and 601 cm^{-1} being characteristic for hydroxyapatite.

The positive and negative shifts of carboxylate peaks can be explained according to their mezomeric structure, showed in figure 5.

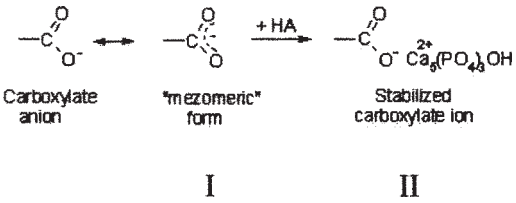

Fig. 5. Mezomeric form stabilization of carboxylate group, due to mineralization

In the absence of HA, carboxylate groups are predominantly in form I, where the two CO bonds are cvasi double (1631.31, 1802.98 cm^{-1}). When HA is present, the carboxylate groups are stabilized and turn into a single and a double bond (1639.76, 1801.22 cm^{-1}). Because of this transformation, one CO bond became stronger and the other became weaker and the corresponding peaks are shifting to higher and respectively lower wave numbers.

Thermal analysis

The ATD (fig.6) curve shows the main thermal effects which occur upon the thermal treatment of the composite samples: between 30 – 200°C an endothermic effect which is due to the water evaporation; between 200 and 600 ^{0}C, the exothermic effect is due to collagen burn.

Consequently, the thermal analysis permitted the evaluation of the composite composition as follows: 8 wt.% water, determined as loss at temperature < 200 ^{0}C; 14 wt.% collagen, determined as weight loss, between 200 and 600^{0}C and 78 wt.% hydroxyapatite, determined by difference.

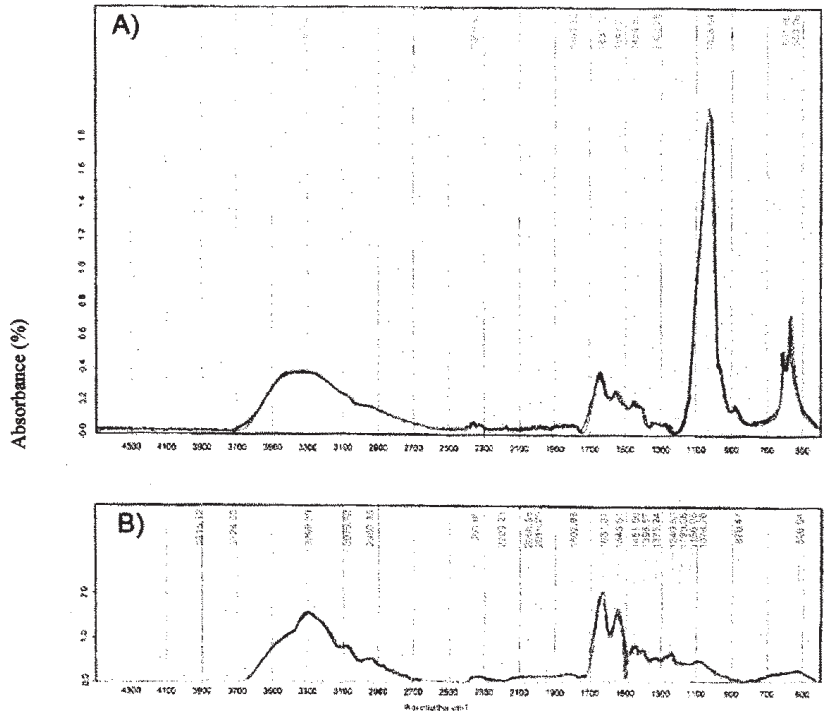

Fig. 4. Infrared spectra of a) pure collagen fibers and b) mineralized collagen fibers

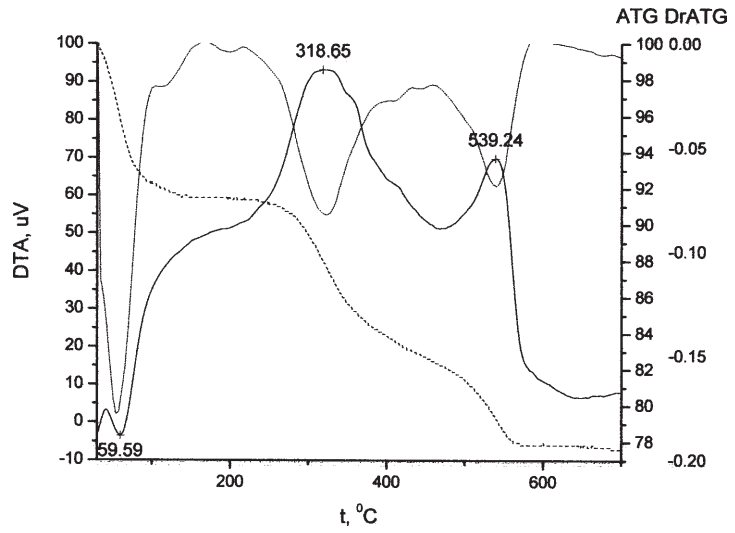

Fig. 6.ATD-TG curve recorded for mineralized collagen fibers, after mineralization and drying

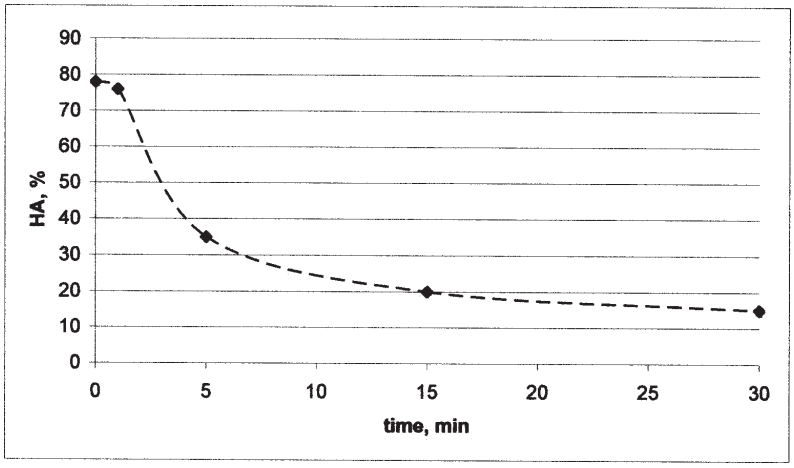

Fig. 7. HA composition in COLL/HA composite during the ultrasonication

In order to quantify the strength of the interaction between the collagen fibres and the hydroxyapatite crystals, the dried mineralised collagen fibers were immersed in water and ultrasonicated for 1, 5, 15 and 30 minutes and consequently rinsed with a large amount of water, in order to remove the weakly attached hydroxyapatite crystals.

Figure 7 shows the influence of ultrasonication on the quantity of hydroxyapatite deposited on the mineralized collagen fibres.

After 5 min of treatment of the water immersed composite with ultrasounds it can be noticed that the proportion of hydroxyapatite in the composite sharply decreases from 80 % to only ~ 34 %. We can assume that the hydroxyapatite conglomerates have been broken during this time, leading to the release of a large quantity of mineral particles. It is well known that nanometric powders exhibit a large tendency to form agglomerates. Further ultrasound treatment leads after another 10minutes to a removal of about 14% hydroxyapatite, and it can be concluded that this corresponds to hydoxyapatite crystals which are more strongly attached to collagen through electrostatic interactions. Even more strong interactions are characterizing the composite material thus obtained, taking into consideration that after 15 more minutes of ultrasonication only approximately 5 % of hydroxyapatite is further removed.

Conclusions

The aim of this work was the investigation of the interaction between pure collagen and hydroxyapatite. A collagen / hydroxyapatite nanocomposite was prepared, by coprecipitation of the mineral phase from hydroxyapatite precursors, on the collagen fibers.

The scanning electron microscopy images showed hydroxyapatite nanocrystals deposited on the collagen fibrils and fibres.

Most probably, the hydroxyapatite nanocrystals are formed due to the nucleation of HA on the collagen material through electrostatic interaction between carboxylate groups and Ca^{2+}. After Ca^{2+} deposition, PO_4^{3-} and HO^- are electrostatically attached.

The X-Ray diffraction pattern proves the formation of nanohydroxyapatite as main mineral phase.

The IR spectra are showing the specific vibrations for hydroxyapatite and collagen. The differences between pure collagen and collagen/nanohydroxyapatite composite are due to the intimate interactions between these.

Through quantitative determinations on samples treated with ultrasounds, it was proved that part of hydroxyapatite particles is strongly attached to the collagen fibers by electrostatic interaction, while other

part is forming agglomerates on the latter, being consequently weaker attached.

The nanocomposite materials prepared are showing an important potential for being used as bone grafts.

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