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Self-assembled collagen/hydroxyapatite composite materials

Anton Ficai[a,*], Ecaterina Andronescu[a], Georgeta Voicu[a], Cristina Ghitulica[a], Bogdan Stefan Vasile[a], Denisa Ficai[a], Viorica Trandafir[b]

[a] *POLITEHNICA University of Bucharest, Faculty of Applied Chemistry and Material Science, Gh Polizu Street 1-7, 011061 Bucharest, Romania*
[b] *National Research & Development Institute for Textiles and Leather (INCDTP) – Leather and Footwear Research Institute, 93 Ion Minulescu st., 031215 Bucharest, Romania*

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ABSTRACT

The purpose of this study is the preparation and characterization of highly oriented collagen/hydroxyapatite (COLL/HA) composite materials, through a self-assembling method, starting from collagen gel and hydroxyapatite precursors by an *in vitro* modified mineralization method. Briefly, this method can be descript as follow: collagen gel (containing 3.21% collagen) is firstly let 24 h in contact with $Ca(OH)_2$ suspension in order that Ca^{2+} neutralize the COOH groups than, in the second stage, the corresponding NaH_2PO_4 is added, in order to form HA. The synthesis of COLL/HA nanocomposite is performed under controlled experimental conditions: 37 °C, pH = 9 and air drying. The morphology of the composite material is strongly influenced by the drying method, especially due to the drying time. If the freeze drying method is used the obtained material is highly porous, but no orientation can be observed. In air, the drying is slow enough so that the wet composite material can reorganize and become uniaxial oriented. Our results prove that in certain conditions, oriented COLL/HA nanocomposites materials can be obtained, starting from collagen and hydroxyapatite precursors, through a very simple and accessible method. It is quite difficult to quantify the orientation degree of the composite, but, most of the fibres are uniaxialy oriented, the average orientation degree being 97.46%. The resulted composite materials were characterized by Fourier Transform Infrared Spectroscopy (FTIR), X-ray diffraction (XRD), Differential Thermal Analysis coupled with Thermal Gravimetry Analysis (DTA-TG), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

1. Introduction

On the US medical devices market the orthopaedics (24%) play a very important role, being advanced only by the cardiovascular sector [1]. Because of the increasing number of bone grafts needed every year and the limited amount of available autografts and allografts, the demand for synthetic bone grafts substitutes increased very much. Comparatively, we can analyse what happened on the US market in 1998 and 2003. In 1998, about 266,000 US patients needed chirurgical intervention for bone grafting [2] and, in 2003, about 500,000 bone restorative chirurgical interventions were reported [3].

Collagen and hydroxyapatite are the main components of the bone tissue [4,5]. The self-assembling process of the organic phase (mainly collagen), mineral phase (mainly hydroxyapatite) and water, gives to bone the special mechanical, chemical and physical properties [6]. The main roles of bone are: to provide the mechanical support for the body, to protect the organs and to act as a mineral reservoir, for Ca^{2+} and $PO_4{}^{3-}$, especially [5,7].

There are two fundamental types of bony tissue: trabecular and cortical. The cortical bone is composed by osteons which are mineralized collagen fibres, disposed parallel each to other. Even if the two types of bony tissue are compositionally similar, due to the different hierarchical structuring the properties are very different. In order to obtain bone-like COLL/HA composite materials, having similar properties with the trabecular bone, the collagen fibres have to be unidirectionally orientated [8,9]. In order to obtain oriented nanostructure a lot of parameters have to be rigorously controlled. The most important parameters which have to be set are: collagen gel concentration and pH; contact time collagen—$Ca(OH)_2$; the adding rate of phosphate; the drying methods and conditions. The influence of pH over the collagen conformation was investigated by Li et al. [10]; they demonstrate that slightly basic media are proper for fibrillogenesis, especially due to the elongated form of the collagen molecules. Gel concentration is very important in order to form collagen fibres. The drying time is very important because the kinetic of fibrillogenesis is quite slow so, long time drying is necessary in order that collagen fibres could be formed. Thereby, free drying is compulsory because when freeze drying is used the wet material is frozen and so the rate of reorganization and drying time drastically decreases and limited orientation occurs.

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[*] Corresponding author. Tel.: +40 74 2902633; fax: +40 21 3181001.
E-mail address: anton_ficai81@yahoo.com (A. Ficai).

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Table 1
Collagen gel characteristics.

Characteristics of collagen gel	Value, wt%	
	Reported to gel	Reported to dry materials
Dry material	3.21	100
Total nitrogen	0.56	1.56
Lipids	–	–
Proteins	3.15	98.13
Ash	0.05	1.56

Even for the same composition, the bones' properties can vary very much with morphology. The most important parameters which influence the properties of bone substitutes prepared in the collagen/HA system are: orientation of collagen molecules, fibrils and fibres that influences the hydroxyapatite structure and orientation; the porosity; the nature and quantity of cross-linking agent and processing methods [5,8,9]. Among these, collagen orientation is the most important parameter which influences the properties of materials, without modifying the composition. This is the reason why many researchers are trying to obtain bone substitutes with oriented structure. In order to mimic the natural bone, many research teams studied the possibility of obtaining COLL/HA composite materials with oriented morphology. In that direction, promising results were obtained using a magnetic field [11], electric field [12] or orientation from a template [13].

Murugan and Ramakrishna [14] classified the bone grafting materials into 4 generations. The forth generation correspond to tissue-engineered (nano)composites bone graft materials [15], the third to the (nano)composite bone graft materials [16,17] while ceramics and polymers are second generation materials [18]. Our research is focused on the synthesis and characterization of COLL/HA composite from the third generation materials. Starting from same precursors, we previously obtained COLL/HA composite materials by layer by layer [19]. Using layer by layer method we proved the possibility to obtain composite materials with different composition and morphology, the collagen fibres which make up the collagen matrix exhibiting no orientation.

In this paper we proposed to improve the COLL/HA composite materials from the third generation by self-assembling. The novelty of this paper mainly consists in the synthesis of the COLL/HA composite materials which had structural and compositional similitude to natural bone. This work presents some points of novelty, the most important being: the synthesis, the methodology of determination of the orientation degree and the mechanism of formation of the COLL/HA composite materials with uniaxial orientation of the constitutive fibres. The self-assembling of the uniaxial oriented mineralized collagen fibrils and fibres is relieved at micrometric (based on SEM images) but also at nanometric level (especially based on TEM and SAED). The mechanism of synthesis of the composite materials with uniaxial orientation of the constitutive fibres was proposed based on the literature data and the obtained experimental results.

2. Experimental

The collagen gel (M.W. = 300,000 Da) was obtained at the National Research & Development Institute for Textiles and Leather, Collagen Department. Calf hide was used to the preparation of collagen, through a special chemical-enzymatic process and purified by dialysis, against water [20]. The pH of the collagen gel is 7.5 while the composition of the collagen gel is summarized in Table 1.

Hydroxyapatite was obtained in situ, in the presence of the collagen gel. The precursors used for HA synthesis were calcium hydroxide (p.a.) and orthophosphoric acid (p.a.), both purchased from FLUKA.

The mineralization process of collagen gel consists of two successive stages. In the first stage, the collagen gel is treated with the desired amount of $Ca(OH)_2$ suspension, drop-wise and magnetically stirred for 24 h and let to interact. In the second stage, the stoichiometric quantity of H_3PO_4 solution was added also drop-wise. $Ca(OH)_2$ was used in order to assure the necessary basic pH. During the first stage of mineralization the pH was maintained at $pH \simeq 9$ by addition of HCl. The ratio of collagen, $Ca(OH)_2$ and H_3PO_4 was so chosen that the final ratio COLL:HA to be 20:80 (wt) and at the end of synthesis the concentration of collagen to became 1.66%. After the H_3PO_4 solution is added, the pH was adjusted at about 9 using NaOH solution, in order to assure pure HA precipitation and the desired morphology will be obtained. During the synthesis and drying of the composite material, the temperature was kept at ~37 °C.

pH 9 was choose based on two characteristics of the collagen molecules:

(a) at pH = 6.9–8 the collagen molecules are in an extended conformation (length of collagen molecule 180–200 nm) and,
(b) the fibrillogenesis is increasing in the range of 6.6–9.2.

The main processes which occur during the synthesis are represented in Fig. 1. The collagen molecules have different conformation, function of the pH can be linear (at $pH \geq 9$) or crimpy (for $pH < 7$); at intermediate pH the collagen molecules being crimpy but with a more pronounced linear aspect. In order to be sure that collagen molecules are in elongated form (linear) the working pH was set at 9.

Function of the mineralization pH, we can obtain different morphologies (Fig. 1b and d). If the pH increases, the crimpy structure of collagen molecules from gel and also from composite can be modified into the elongated one. If the drying time is slowly enough, these linear (elongated) collagen molecules can self-assembly and form cylindrical fibrils and fibres (Fig. 1e), otherwise they will form fibrils and fibres, growth tri-dimensional, with crimpy collagen molecules (for $pH < 7$) or with linear collagen molecules (for $pH \geq 9$). The structure presented in Fig. 1b can be transformed into the structure presented in Fig. 1d by increasing of pH at an adequate value. In this case, the pH must be higher than in case of transformation of the structure illustrated in Fig. 1a into the structure presented in Fig. 1c, probably due to the interaction between HA and collagen.

After synthesis and drying, the composite material was analysed by: X-ray diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Differential Thermal Analysis coupled with Thermal Gravimetric Analysis (DTA-TGA).

X-ray diffraction analysis was performed using a Shimadzu XRD 6000 diffractometer at room temperature, using Cu Kα radiation. The samples were scanned in the Bragg angle, 2θ range of 10–70, with a scanning rate of 2 °C/min.

SEM analyses were performed on a HITACHI S2600N electron microscope with EDAX, on samples covered with silver layer.

The transmission electron images were obtained on finely powdered samples using a Tecnai^{TM} G^2 F30 S-TWIN high resolution transmission electron microscope (HR-TEM) equipped with STEM—HAADF detector, EDX and EELS. The microscope was operated in transmission mode at 300 kV while TEM point resolution was 2 Å and line resolution was 1 Å. The hydroxyapatite particle formed on the collagen fibrils was assessed by selected area electron diffraction (SAED).

For IR measurements, Brucker—VERTEX V70 spectrophotometer was used. The spectra were recorded over the wave number range of 400–4000 cm^{-1} with a resolution of 2 cm^{-1}.

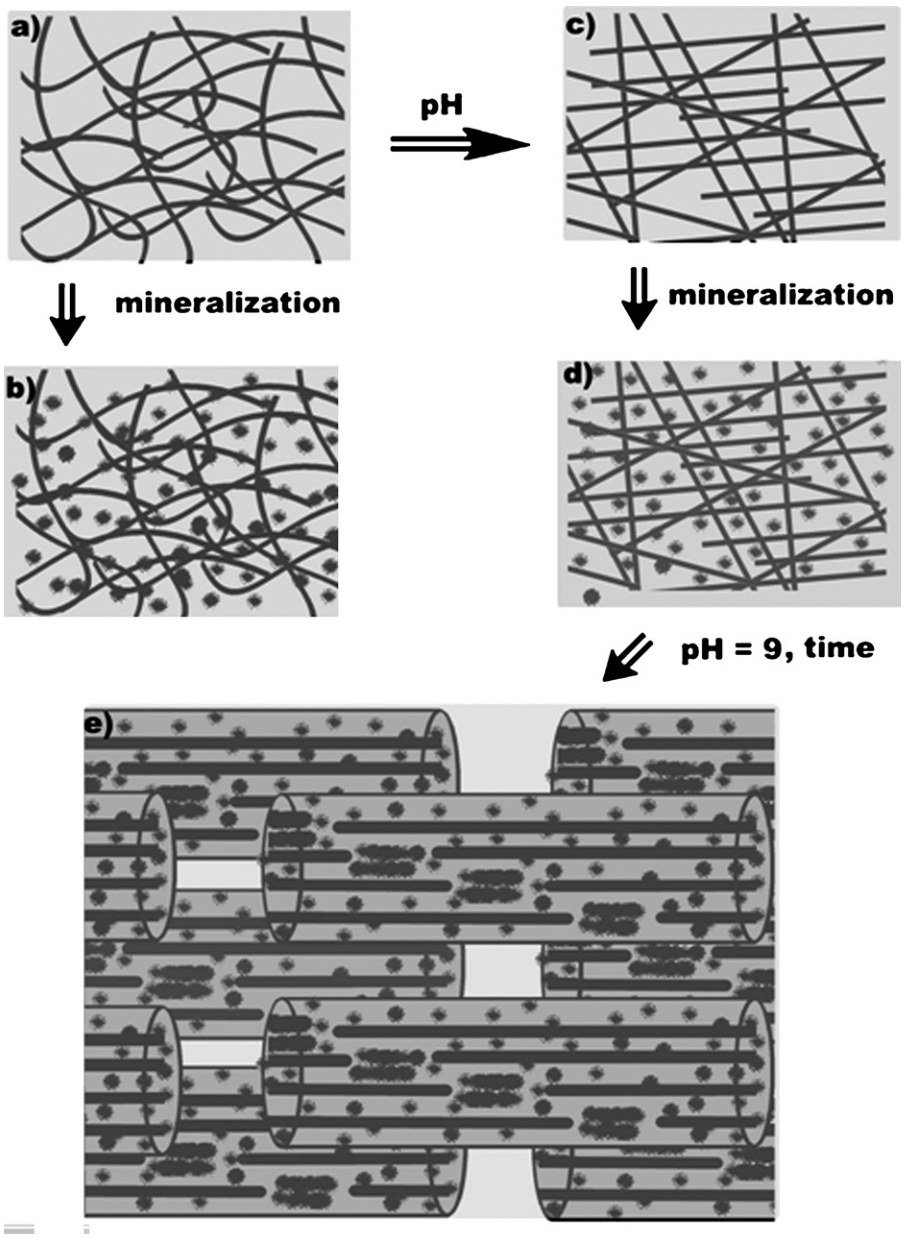

Fig. 1. Specific processes occurred in solution during the self-assembly of COLL/HA composite materials.

The differential thermal analysis (DTA) coupled with thermo gravimetric analysis (TGA) was performed on a Shimadzu DTG-TA-50H, at a heating rate of 10 °C/min, in static air.

3. Results and discussion

XRD was used to evidentiate the mineralization process. The XRD spectrum, in Fig. 2, shows the formation of HA.

In the case of COLL/HA composite obtained by self-assembly comparing with the HA obtained by precipitation, the X-ray diffraction pattern is evidenciating a much higher intensity for the 2 1 1 peak reported to the intensity of the other peaks characteristic to HA. This result can be attributed to a preferential growth of the HA crystals in the 2 1 1 direction due to the collagen influence. As it can see, the composite material also contains NaCl (Fig. 2a). The removal of NaCl can be easily made by washing the composite materials with distilled water, following the next procedure: after drying, the COLL/HA composite material with uniaxial orientation of the constitutive fibres is crosslinked with glutaraldehyde, washed with plenty of water and dried. Following this procedure, the chloride was completely removed (Fig. 2b) without altering the mineral phase, especially from the point of view of crystallinity and preferential crystallization direction.

The self-assembling structure of collagen molecules and hydroxyapatite particles can be proved by Scanning Electron Microscopy (Fig. 3). The samples were analysed in perpendicular and parallel section reported to mineralized collagen fibres, in order to study the formation and the orientation of collagen fibrils and fibres. The sodium chloride removal not alters the composite morphology; the SEM images recorded before and after sodium chloride removal being similar.

Whatever the analysis section, the recorded SEM images show the formation of collagen fibrils and fibres, which are mineralized with HA.

The SEM image presented in Fig. 3a is recorded in a fibres perpendicular section. This image shows a homogenous arrangement of the fibres. Fig. 3b and c is recorded in a fibres parallel profile and shows, at different magnification the stratified structure of the

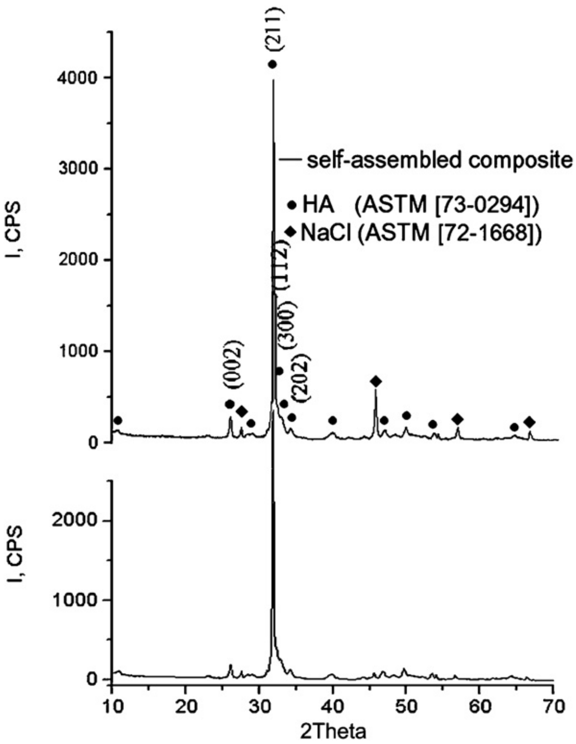

Fig. 2. XRD pattern of COLL/HA composite material (a) before sodium chloride removal and (b) after sodium chloride removal.

composite materials, the fibrils and fibres being organized in layers. It can also observe highly oriented fibres and fibres bundle. The homogenous arrangement can be evidentiated also in fibres parallel profile. Analyzing the rupture profile, it can be observed a shift between the rupture point, comparing different neighbour adjacent layers. In Fig. 3c, recorded at higher magnification, it can better observe the mineral deposition. It can conclude, that at the end of collagen fibres, the HA density is higher. At broken, the rupture seems to follow the highly mineralized zone of interfibrilar gap which exist at the end of two successive fibres. Finally, at a much higher magnification (Fig. 3d) the SEM images of fibres' end are evidentiating the dimensions of HA in the nanometric range, being between a few nanometers and up to 40 nm.

At high magnification from SEM image, it can be seen that the dimensions of the HA crystals are in the nanometric range (4–40 nm), with an elongated morphology. These results are in good agreement with the observation reporting to natural HA from the natural bone.

The SEM images obtained at different magnification, in the rupture, on parallel and perpendicular direction of collagen fibres, are showing a homogenous microstructure, with fibres organized in layers, with high orientation. Taking also in consideration the way in which the rupture took place, and also the fact that we have a high concentration of HA grains in the rupture points we might conclude that the COLL/HA composite material synthesized has a very similar structure with that of the natural long bone, that have been obtained through auto-assembling process.

Obviously the orientation is difficult to quantify and only a few methods permit this kind of measurements (and usually these are indirect methods); the orientation will be quantify based on the SEM images.

In order to quantify the orientation, we choose the "*c* axis" such as to obtain a minimum of $\sum_{i=1}^{N} DA^2$. In these conditions, the average deviation of fibres can be calculated as follow:

$$\text{3D systems}: \bar{D}(\%) = \sqrt{\sum_{i=1}^{N}\left(\frac{\sqrt{\alpha^2+\beta^2+\gamma^2}}{N}\right)^2}\,\frac{100}{90}$$

where $\bar{D}$ is the average deviation of each fibre from the unidirectionality; N is the number of fibres; 100/90 represent a correction factor in order to obtain the average deviation factor as percent; and α, β and γ are the deviation angle of each fibres (assumed as a straight line) into the three dimensions as that showed in Fig. 4. The deviation angle, in all the three dimensions, used in the above formula, can be easily determined, as follow:

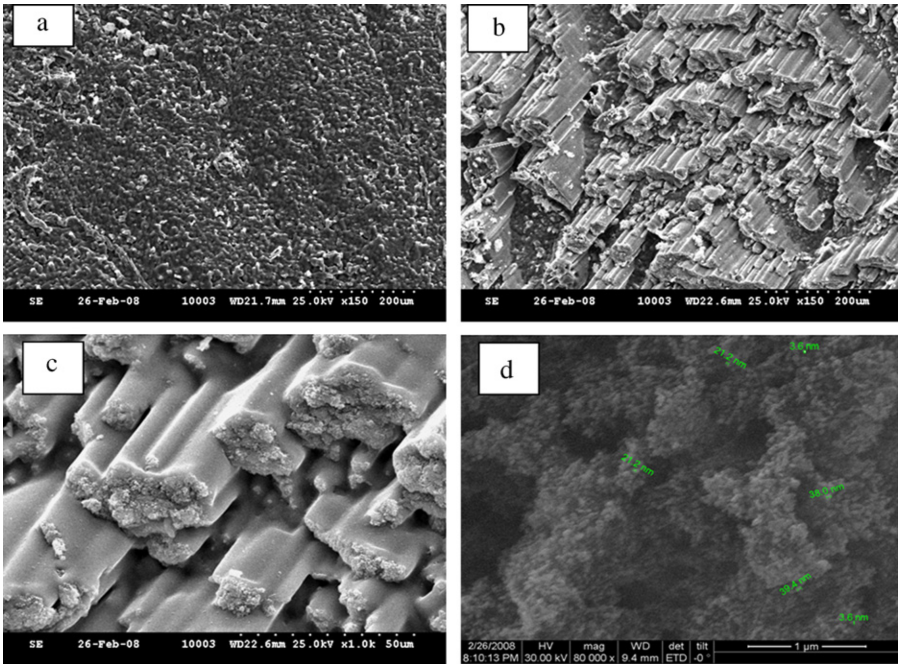

Fig. 3. The SEM images of collagen/hydroxyapatite composite materials, recorded at different magnification; (a) parallel view with the fibres, (b–c) perpendicular view with the fibres, (d) high resolution SEM at 80,000× magnification.

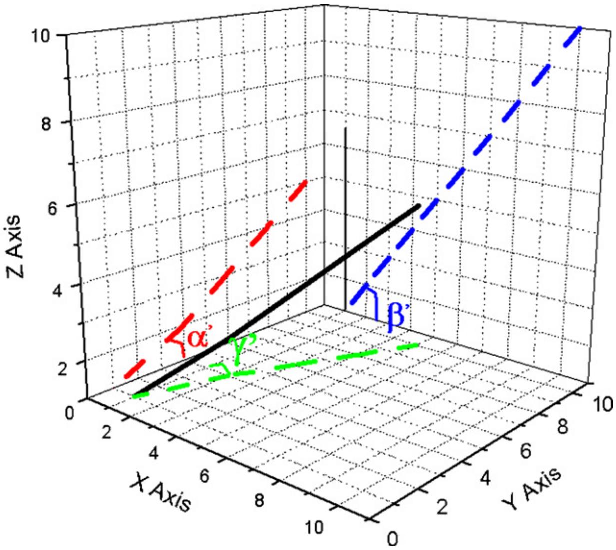

Fig. 4. Straight line projection onto the three dimensions and visualization of the three angles formed between the projections and the three dimensions.

- if $90 < \alpha'$, β' or $\gamma' \leq 180$ than α, β or $\gamma = 180 - \alpha'$, β' or γ';
- if $180 < \alpha'$, β' or $\gamma' \leq 270$ than α, β or $\gamma = \alpha'$, β' or $\gamma' - 270$;
- if $270 < \alpha'$, β' or $\gamma' \leq 360$ than α, β or $\gamma = \alpha'$, β' or $\gamma' - 360$;
- if $0 < \alpha'$, β' or $\gamma' \leq 90$ than α, β or $\gamma = \alpha'$, β' or γ'.

Based on the above mentioned and the SEM image presented in Fig. 3b the average deviation of each fibre was calculated based on a 2D model and is $2.54 \pm 0.2\%$ ($\gamma = 0$ because SEM is a 2D method).

It is worthy to note that because of the preparation of the sample collagen fibres are destroyed and only the collagen molecules and fibrils will be possible to visualize by TEM (Fig. 5); the collagen molecules and fibrils being in elongated form, associated with hydroxyapatite nanoparticle. The selected area electron diffraction recorded for an area of $\sim 1\ \mu m^2$ is in good agreement with the XRD pattern recorded for the finely powdered material.

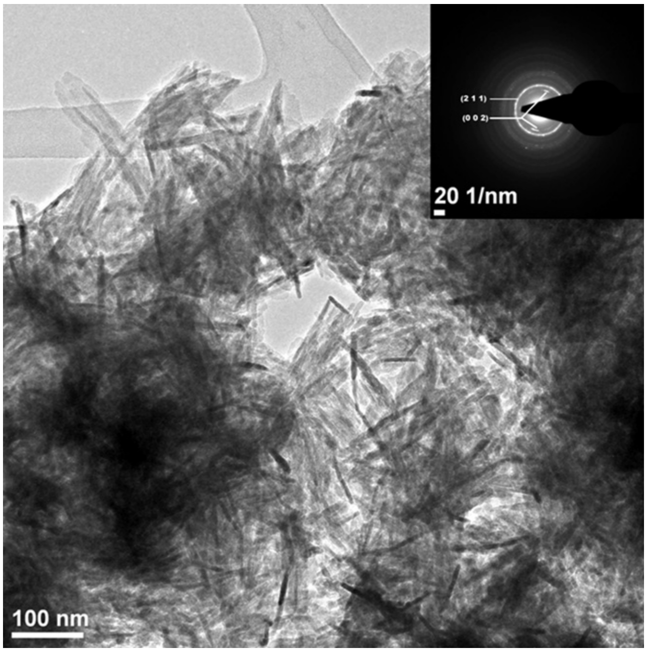

Fig. 5. TEM image of mineralized COLL/HA composite material; the insert correspond to selected area electron diffraction.

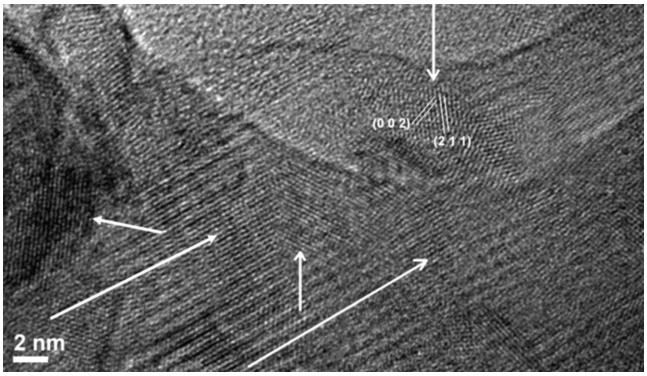

Fig. 6. HR-TEM image of mineralized collagen fibrils. The long arrows indicate the collagen fibrils while the short arrows indicate the nanoHA grains deposited on the collagen or at the end of collagen fibrils.

HR-TEM analysis is done in order to study the interaction between the collagen fibrils and the HA nanoparticles. The HA particle has an average dimension of a few nanometers and can be identified on the collagen fibrils (molecules) but also at the end of them. Based on these images it can be assert that collagen and hydroxyapatite lead to a composite material with a good similarity with the natural bone as described by Cui et al. [21,22] (Fig. 6).

Based on the work published by Kikuchi et al. [23] the degree of self-assembly can be estimated based on the fibres length and the crescent corresponding to selected area electron diffraction pattern of HA, 002; the degree of self-assembling being very good.

The two main processes which occur upon thermal treatment are evidentiated by DTA and TG (DTG) curves (Fig. 7) water loss (endothermic process accompanied by mass loss, with maximum rate at 65 °C) and collagen burning (exothermic process accompanied by mass loss, realized in two-steps, with maximum rate at 325 and 429 °C). Subsequently, it can estimate the composite material composition: the water content is of about 5%, the collagen is about 18%, while the proportion of mineral phase is reaching about 77%.

It is worthy to mention that the temperature of the two-step decomposition process of the composite studied here are very close to those reported by Lozano et al. [24] for the human bones decomposition indicating a similar behavior among our composite and the human bones.

The infrared spectra (Fig. 8) of obtained COLL/HA composite shows the characteristic peaks corresponding to collagen

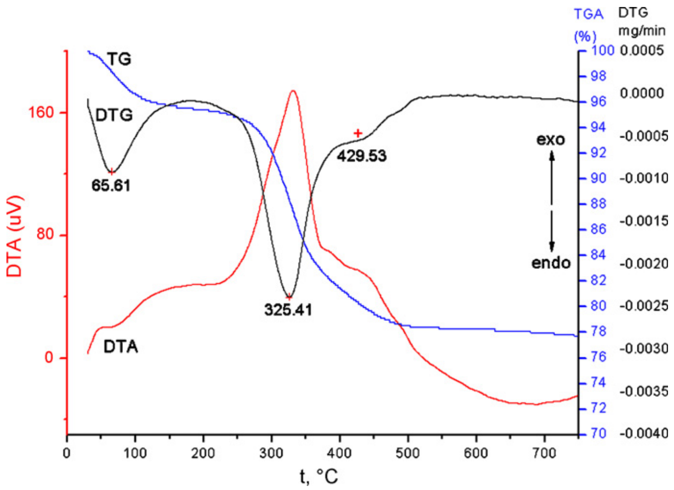

Fig. 7. DTA-TG curves recorded for COLL/HA composite.

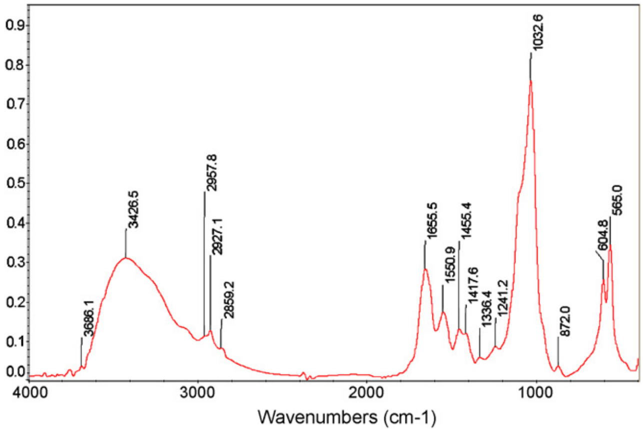

Fig. 8. Infrared spectrum of COLL/HA composite material.

and hydroxyapatite. The spectral attribution can be easily made, according with the literature data [4,25].

The characteristic peaks of hydroxyapatite were determined by deconvolution: ν_1 P–O symmetric stretch (957 cm^{-1}); ν_4 O–P–O bend, triple degenerate (565, 605, and 641 cm^{-1}); ν_3 P–O antisymmetric stretch, triple degenerate (1030, 1065, and 1110 cm^{-1}). As the natural bone, the obtained COLL/HA composite materials, contain carbonated apatite, the presence of carbonate being proved based on the absorption bands from 1336 (ν_3 $CO_3{}^{2-}$) and 872 (ν_2 $CO_3{}^{2-}$).

For collagen, the characteristic peaks are: 1650 cm^{-1} corresponding to amide I, C=O; 1550 cm^{-1} corresponding to amide II (N–H stretch, C–N bend), 1417 cm^{-1} corresponding to C–N bend; 1241 cm^{-1} corresponding to amide III (C–N bend, N–H stretch), 2859 cm^{-1} $\nu(CH_2)$ asymmetric; 2927 cm^{-1} $\nu(CH_2)$ symmetric; 2957 $\nu(CH_3)$ symmetric.

The wide peak at 3000–3500 cm^{-1} corresponds to associated OH.

The integrity of the collagen from the composite material was proved based on the method described by Silva et al. [26], and was reconfirmed by the unaltered ratio between the peaks intensity of the composite material and pure collagen from 1450 and respectively from 1240 cm^{-1}.

The obtained results have proved the formation of a COLL/HA composite materials, with a highly oriented structure, that can be due to auto-assembling process, which took place during synthesis.

Many research studies are dedicated to self-assembling of different materials, many of them involving proteins [27,28]. The self-assembling process can be realized as a result of many kind of interactions between the two (or more) phases. In COLL/HA composites, the most important interactions involved are electrostatic interaction between opposite charged components or hydrogen bonding. These interactions can occur between hydroxyapatite grains, between different collagen hierarchical structures, but also between the organic and the inorganic phases. The most important interactions which occur in COLL/HA composites are presented in Fig. 9 [29].

Hydrogen bond occurs inside the collagen molecule (between the three constituents polypeptides), between collagen molecules (as shown in Fig. 9), but also between collagen fibrils and fibres. Also, hydrogen bond occurs in hydroxyapatite and between collagen and hydroxyapatite.

Electrostatic interaction occurs especially between Ca^{2+} cations (from hydroxyapatite) and carboxylate groups (from collagen) and is at full discussed in a previous paper, based especially on FTIR and SEM analysis [30] and between different hydroxyapatite grains (especially between Ca^{2+} and phosphate anions). Also electrostatic interactions occur inside collagen molecules, but also between collagen fibrils and fibres (between charged groups such as carboxylate or phenolic groups and positively charged ammonium groups from arginine, for example).

If we accept that the self-assembling of collagen fibrils and fibres and HA particle are the same as in collagen molecules and HA, the schematic representation of collagen–HA assembling presented in Fig. 9, can explain the failure of the material (Fig. 3b–d).

The obtained composite structure is in good agreement with the structure proposed by Hellmich et al. [31]: mineral matrix consisted in HA is reinforced with mineralized collagen fibrils and fibres. The mineralized collagen fibres are formed from mineralized collagen fibrils. The fibrils diameter is in the range of some hundreds nanometers and up to a few micrometers while the fibres are in the range of some micrometers and smaller than 100 μm.

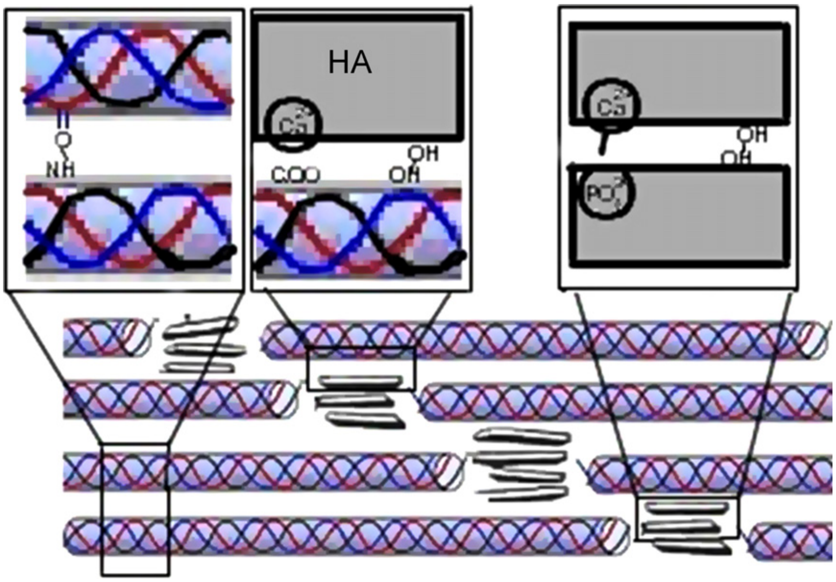

Fig. 9. Interaction types in COLL/HA composites.

4. Conclusion

The purpose of this study was to obtain and characterize the COLL/HA composite materials, which mimic the natural long bone.

Hydroxyapatite was obtained by co-precipitation in the presence of collagen gel. In fact, starting from collagen gel and hydroxyapatite precursors, in certain conditions, due to the interactions between collagen, hydroxyapatite and water it was obtained self-assembled, highly oriented composite materials. It can note that, many authors published a lot of papers dealing with COLL/HA composite materials which start from collagen gel and calcium hydroxide and ortophosphoric acid as precursors, but due to the inadequate processing conditions they do not obtained the uniaxial orientation of the constitutive fibres.

In aqueous solution the collagen molecules and hydroxyapatite precursors have the capacity to induce synthetic bone formation by self-assembling. The obtained composite material is more similar with compact bones morphology; the recorded analysis being very similar with these bones.

Very important is that we also propose a new way to estimate the average deviation of fibres and we determined it, assuming a 2D model. The average deviation is $\sim 2.54 \pm 0.2\%$ which means that the degree of orientation is $97.46 \pm 0.2\%$.

The area of knowledge in the field of COLL/HA composites materials orientation was improved by assisted self-assembling.

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