The Influence of Ultrasonication on the Formation of COLL/HA Composite Materials

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The use of ultrasonication can be an useful tool to improve the properties of the collagen/hydroxyapatite (COLL/HA) composite materials. The aim of this paper is to study the influence of the ultrasonication on the synthesis and characteristics of the COLL/HA composite materials. Especially based on the microscopic analyses it can be observed a more uniform distribution of the HA, practically the HA covering the collagenous materials. The integrity of collagen was studied by FTIR, comparing the COLL/HA composite spectrum of the sample obtained by ultrasonication with that of the control sample (no ultrasonication was applied). Due to no changes observed at the C-O region and due to the unaltered ratio between the peaks intensity (between the intensities of the peaks from 1450 and respectively 1240cm^{-1}) of the two composite materials it can be asserted that ultrasonication does not alter the structure of collagen. The resulted composite materials were characterized by Fourier Transform Infrared Spectroscopy (FTIR), X-Ray Diffraction (XRD), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

Keywords: collagen/hydroxyapatite nanocomposite; transmission electron microscopy (TEM), ultrasonication

Due to the increasing need of bone grafts many new (nano)materials were obtained in order to be tested and used as bone substitutes. Due to the similarity with the natural bone, COLL/HA composite materials are extensively studied for their potential use as bone substitutes [1-4]. Also, many natural polymer/calcium phosphates [5-9] or many other types of materials, including composite [10-12] or monolithic [13-15] materials were studied as bone substitutes.

The synthesis of new nanomaterials with improved properties is a continuous challenge of the researcher. The interest of the researcher for this domain leads to an increasing number of journals and papers published yearly. As a general trend of this era, the synthesis of the nanostructured bone grafts became reality. First paper dealing with "nano" "bone grafts" was published in 2001 [16]. Still there were published thousand of papers, especially in the last years. The nanostructured materials exhibit good alternates for bone tissue engineering [17-20].

Ultrasonication was previously used for different applications, some of the most common being listed below.

Ultrasonication is worldwide used in order to obtain dispersed nanoparticles starting from agglomerates [21].

As a conclusion of their work, the researchers presented the influence of the ultrasonication on the morphology of the synthesized lanthanum phosphate [22]. The use of ultrasonication allows the synthesis of lanthanum phosphate under controlled conditions. For instance, at *p*H=1 the use of ultrasonication leads to the formation of nanorods (5-9 nm wide and several tens to several hundreds nm long) while the control samples, obtained without ultrasonication lead to the formation of a mixture of nanoparticles and nanorods. At *p*H=12 spherical nanoparticle were obtained (about 5nm or less).

Ultrasonication can assist also in many chemical reactions [23-25]. For instance, the ultrasonication can enhance or promote chemical oxidation of fullerenes [25] or can induce sulfation of curdlan [24]. Between the reactions induced by ultrasonication also degradative processes of natural polymers were observed [23].

The main point of novelty consists in the synthesis of the composite materials simultaneously in the presence and in the absence of the ultrasonic field. Because ultrasonication can induce degradative processes of natural polymers, collagen integrity had been studied.

Experimental part

Synthesis of COLL/HA composite materials

Collagen gel 1,6% (*p*H = 3.2) was used as received from Footwear Research Institute–Collagen Department. The molar weight of the used collagen is 300 kDa.

Hydroxyapatite was obtained in situ, by co-precipitation, starting from $Ca(OH)_2$ and $Na_2HPO_4 \cdot 12H_2O$, both purchased from Fluka. The mineralization process was conducted in two stages, in the first stage the calcium hydroxide suspension is added to the collagen gel and let to interact 24h while in the second stage the phosphate solution is drop wise added. The resulted slurry was let 24h for the HA maturation at *p*H=~9. Both stages as well as the maturation process were conducted under ultrasonication. The reaction temperature was maintained at 37^{0}C during the synthesis. The ratio between collagen, calcium hydroxide and phosphate was as chosen to obtain the COLL: HA ratio of 1:4. Simultaneously with the composite obtained in the ultrasonication field a blank sample was obtained in the same conditions but, without ultrasonication.

The as obtained two composite materials were dried by freeze-drying.

COLL/HA composite materials characterization

The obtained materials were investigated by XRD, FTIR, SEM and TEM.

X-ray diffraction analysis was performed using a Shimadzu XRD 6000 diffractometer at room temperature,

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on finely powdered materials. In all cases, Cu Kα radiation from a Cu X-ray tube was used.

SEM images were recorded on a HITACHI S2600N electron microscope coupled with an EDS detector. Prior analyses, all samples were covered with silver layer by plasma sputtering.

For IR spectroscopy (*Shimadzu 8400* FT-IR Spectrometer) measurements, the spectra were recorded in the wave number range of 500 – 4000 cm^{-1}, with a resolution of 2 cm^{-1}.

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

Results and discussions

The characterization of these materials was made from the point of view of morphology of the composite material, the structural integrity of the collagen as well as the thermal behaviour of the COLL.HA composite obtained by ultrasonication.

X-Ray Diffraction

X-ray diffraction was recorded to study the influence of the ultrasonication especially on the crystallinity and crystallite size of the obtained HA. It can be seen that ultrasonication leads to an unidirectional crystallized hydroxyapatite (211) similar to that obtained by self-assembling [26]. Based on Scherer equation the mean size of the crystallite is 30 nm.

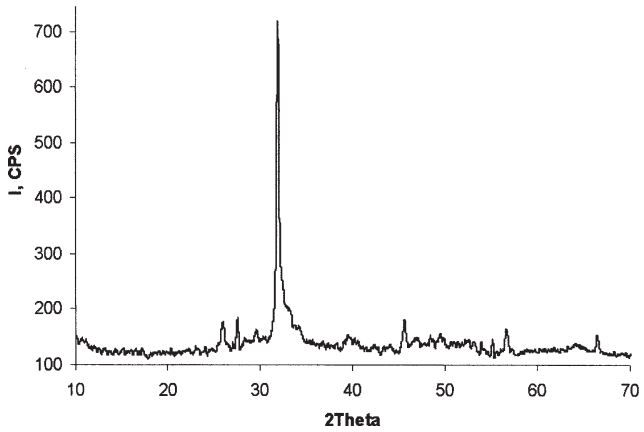

Fig. 1. XRD pattern of COLL/HA composite material obtained by ultrasonication

Scanning electron microscopy

The SEM images recorded at any magnification confirm a compact structuring of the COLL/HA composite material (fig. 2). In the case of the composite material obtained by ultrasonication the mineral phase cover very well the collagenous support and permit to analyze it at higher magnification, even 12.000x which is not possible in the case of COLL/HA composite materials obtained without ultrasonication. The morphology of the composite material obtained by ultrasonication is similar regardless the magnification.

At high magnification, the dimension of the HA agglomerates was determined, the HA agglomerates having less than 250 nm.

Energy dispersive spectroscopy

Energy dispersive spectroscopy was used only, in order to study the Ca/P ratio. In both case, this ratio varies

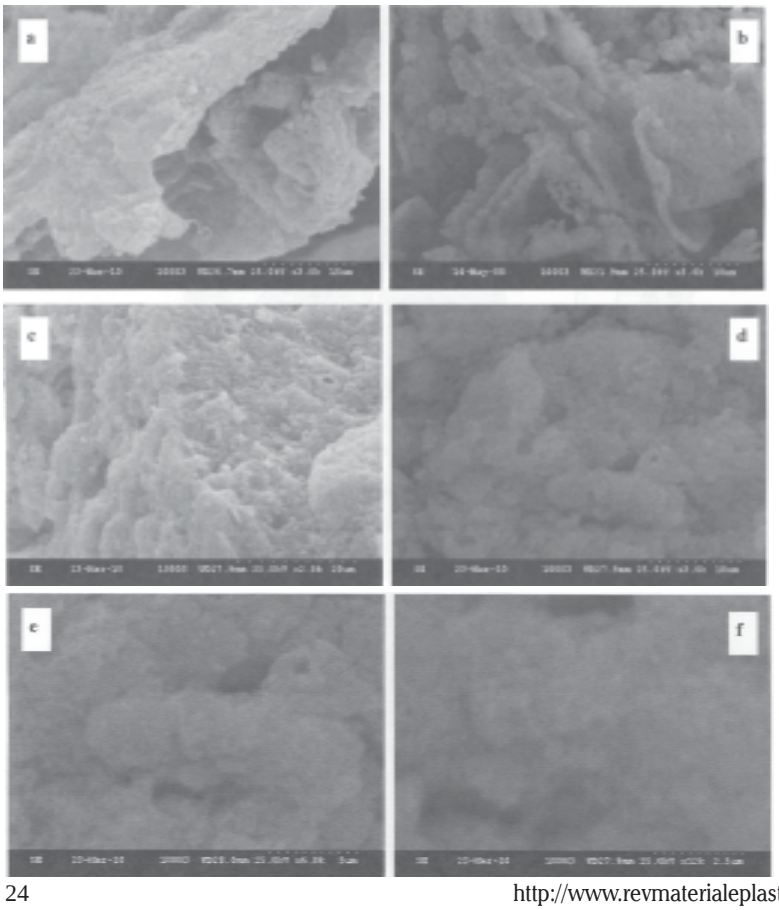

Fig. 2. SEM images of COLL/HA composite materials obtained a-b) without and c-f) with ultrasonication, at different magnification

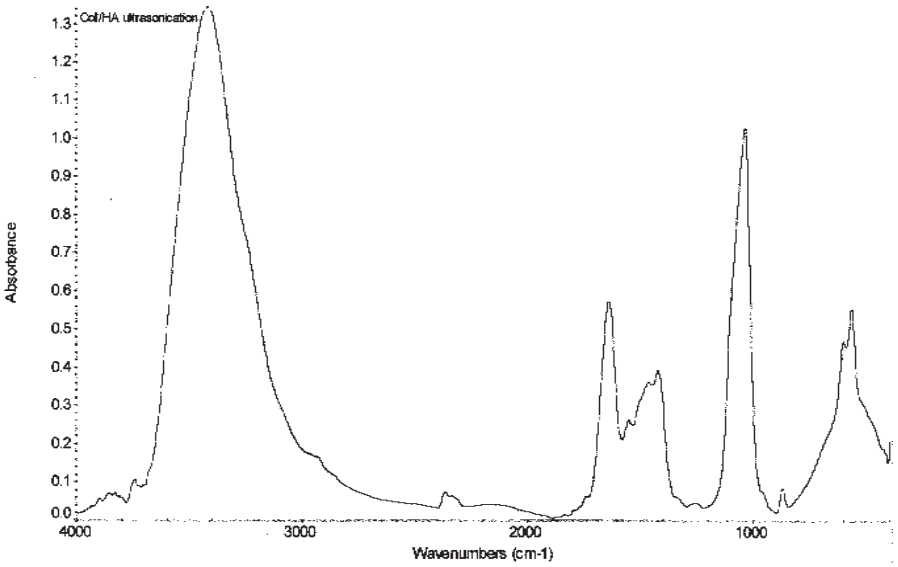

Fig. 3. FTIR spectrum of COLL/HA composite materials obtained by ultrasonication

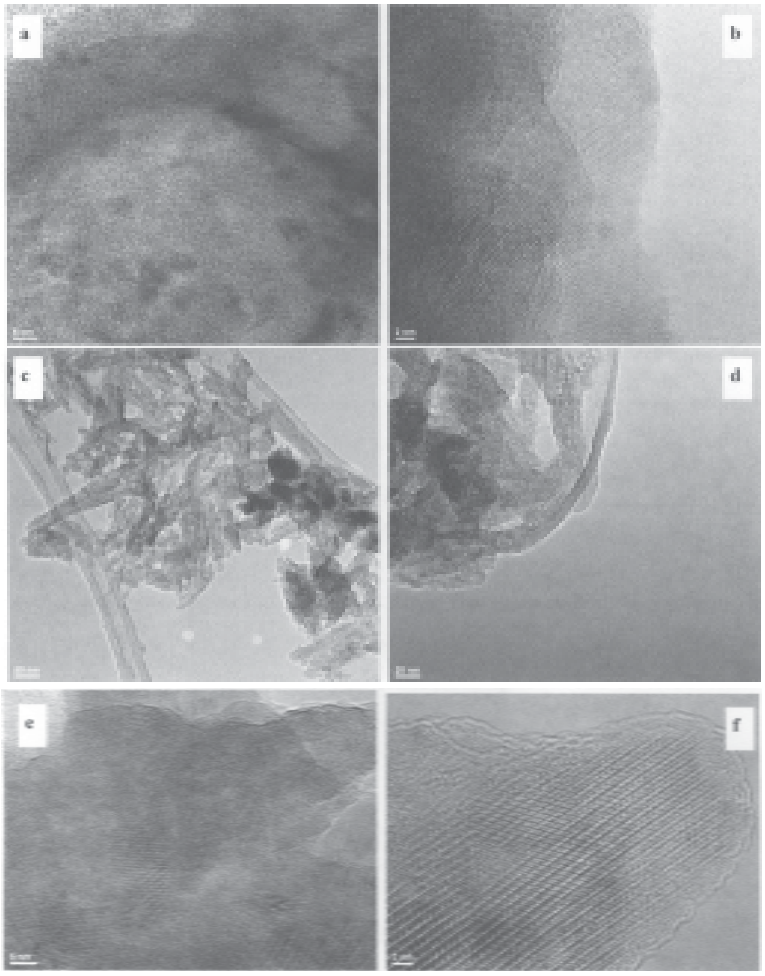

Fig. 4. TEM and HRTEM images of COLL/HA composite materials obtained a-b) without and c-f) with ultrasonication, at different magnification

between 1.64 and 1.68 being not influenced by ultrasonication.

Infrared spectroscopy

Infrared spectrum (fig. 3) is similar for both composite which mean that the ultrasonication did not induced any structural modification. The main absorption bands attribution of the components can be made according to our previous work [26].

Transmission electron microscopy

The main differences which appear in transmission electron microscopy between the two kinds of COLL/HA composite materials are at nanometric level. The TEM images recorded for the COLL/HA composite material obtained by ultrasonication permit the visualization of the HA nanoparticles deposited on the mineralized collagen support as we can see at high resolution. Also, by TEM the characteristic, fibrillar shape of collagen type I can be identified (fig. 4c, d).

The two materials obtained with and without ultrasonication were also analyzed by HRTEM. In the case of COLL/HA composite materials obtained by ultrasonication a higher crystallinity of the mineral phase can be seen and, the mineral phase covers perfectly the collagenous support (fig. 4f). In the case of the control sample (figl. 4a, b) especially solitary HA nanoparticles can be visualized onto the collagenous support.

Based on the TEM and HRTEM images recorded for the COLL/HA composite materials obtained by ultrasonication two kinds of HA can be identify. Firstly, at low resolution

HA particles can be identifying (fig. 4c, d) which is deposited on a thin HA layer covering the organic phase (fig. 4e). The HA nanoparticle can reach up to 10nm as results from figure 4d while the thin film can reach even 100nm as it can be estimated based on figure 4e, f.

Conclusions

First of all, due to the ultrasonication the HA is preferentially deposited onto the colagenous support instead of to form agglomerates. The synthesis of COLL/HA composite materials by ultrasonication leads to COLL/HA composite materials with improved properties.

Based on FTIR analysis the ultrasonication does not induce collagen degradation; all characteristics absorption bands of each components being unaltered.

Very important is that the ultrasonication induces a preferred crystallographic orientation (211) of the hydroxyapatite.

Due to these special deposition of the HA, which form a thin ceramic film on the surface of collagenous support, this composite material exhibits better stability into the electron beam in both SEM and TEM measurements, HA acting as a "protector" for the organic phase. HA can be found in two distinct hypostasis, as a thin HA layer covering the organic phase and, as nanocrystals deposited onto the HA layer.

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