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BIOMIMETIC MINERALIZATION OF THE HUMAN NASAL SEPTUM CARTILAGE

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Simulated body fluid (SBF) immersion technique is an attractive method for biomaterial synthesis due to the conditions similar to the *in vivo* synthesis ones. The collagen/hydroxyapatite (COLL/HA) composite biomaterials obtained by immersing the collagen matrices in SBF are usually well mimicking the bone, especially the mineral phase synthesis and properties. Starting from these hypotheses, we have achieved the mineralization of human nasal septum cartilage by SBF immersion technique. For this, nasal septum cartilages were collected from a 33 years old woman and used as obtained. The mineralization was conducted by immersion for 168 hours at ~37^{o}C and pH=7.4. The cartilage mineralization process by SBF immersion can be compared with the endochondral ossification occurring naturally. When compared with other related materials, the obtained materials have shown better characteristics because the organic phase had been synthesized by the human body and, the mineral phase was deposited by a biomimetic method. Summarizing, the proposed method can be considered an advanced biomimetic method and the obtained materials can be expected to exhibit improved properties.

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1. Introduction

The SBF immersion technique is one of the most suitable methods for the synthesis of collagen/hydroxyapatite composite materials [1, 2]. This method consists in the immersion of the collagen matrices into SBF [3]. The quantity of mineral phase that can be deposit is dependent on the immersion time, temperature, pH and many other factors. Usually, the SBF is obtained by dissolving the proper salts, in proper ratio, in distilled water [4].

SBF immersion technique was used successfully for the synthesis of new materials [3, 5, 6]; to induce a pre-mineralization process of materials [7], and to improve some materials obtained by other conventional methods [8-11]. For instance, HA based materials obtained by co-precipitation method were improved by SBF immersion, the resulting materials showing higher biocompatibility [12].

Collagen is the main component of the extracellular matrix of many tissues, the most important collagen-based tissues of human and animals body are: skin, cartilages, tendons and bones.

A cartilage consists of a fluid phase and a solid phase [13]. The fluid phase consists mainly of water with physiological concentrations of naturally occurring ionic and non-ionic

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solutes of body fluid, the fluid phase representing ~75–85% of the wet cartilage [14]. As compared to the wet cartilage, the solid phase consists of chondrocytes, collagen, proteoglycans (aggrecan), lipids, and small amounts of glycoproteins [15].

Bones are formed *in vivo* through two distinct developmental processes: endochondral and intramembranous ossification. The endochondral mineralization involves an intermediate cartilaginous phase formation and its mineralization. As a result of endochondral mineralization the long bones, facial bones and lateral clavicles are formed [16].

Also, many abnormal mineralizations of hard tissue or of many soft tissues were reported in the literature. Extraosseous calcification (many times real ossification process) includes arteriolopathy, solid organs calcification, corneal, peritoneal, vascular and/or valvular calcification [17-20].

The aim of this study was to realize the conversion of the natural, human cartilage into bone like material. The conversion of the human nasal septum cartilage to a bone like material has been achieved by cartilage mineralization by SBF immersion technique.

2. Experimental

Human cartilage was obtained from a 33 years old woman who underwent an operation to correct the nasal septum deviation. The cartilages were rinsed with plenty of demineralized water and mineralized immediately after the operation by SBF immersion technique.

The mineralization process occurs by immersing the cartilage (0.2g) into the simulated body fluid (SBF-1L) at 37^0C for 168h. The pH value was adjusted to 7.4 by means of the TRIS buffer (*tris* (hydroxymethyl) aminomethane and *tris* (hydroxymethyl) aminomethane hydrochloride – TRIS·HCl). The major species concentrations in the SBF were found to be similar to those of the human plasma, except chloride and phosphate. The higher phosphate concentration has imparted to the SBF solution the human cartilage mineralization ability [21].

The collagen matrix was obtained in the Collagen Department of the National Research & Development Institute for Textiles and Leather – Leather and Footwear Research Institute, starting from bovine collagen gel by crosslinking and freeze drying. The collagen gel (type I, M.W. = 300 kDa) was obtained by extraction from bovine calf.

After mineralization all the samples were freeze dried in a Christ Alpha 2.4 freeze drier.

X-ray diffraction analyses were performed using a Shimadzu XRD 6000 diffractometer at the room temperature. The samples were scanned in the Bragg angle, 2θ range of 10–70^o at a scan rate of $2^o min^{-1}$.

Scanning electron microscopy images were obtained with a HITACHI S2600N microscope coupled with an EDS probe. All samples were covered with a silver layer prior to imaging.

Infrared spectroscopy (IR) measurements were performed using a Vertex 70 instrument (Brucker) with Fourier transformation (FTIR), equipped with ATR module based on diamond crystal. The spectra were recorded over the wavenumber range of 400–4000cm^{-1} with a resolution of 2 cm^{-1} for the human cartilage as well as for mineralized cartilage.

Differential thermal analysis (DTA) coupled with thermogravimetric analysis (TGA) was performed in the air atmosphere with a Shimadzu DTG-TA-50H, at a heating rate of 10^oC/min, in the static air.

3. Results and discussions

The nasal septum cartilage and the mineralized cartilage were analyzed for their morphology and composition by XRD, FTIR, SEM and DTA-TG.

3.1. X-Ray diffraction

Based on the literature data [22] the mineral components of cartilage are those derived from the body fluid and are presented in Figure 1. The examination of the XRD cartilage pattern (Figure 1a) reveals the peaks corresponding to NaCl and Na_2CO_3 / $NaHCO_3$ while the other

components cannot be identified because of the very low concentration and crystallinity. The broad peak centered at about 20^o corresponds to the crystalline form of the organic mater from the cartilage. Based on Scherrer equation the size of the main crystallites is 16-20nm characteristic to the well crystallized components such as NaCl, Na_2CO_3 or $NaHCO_3$.

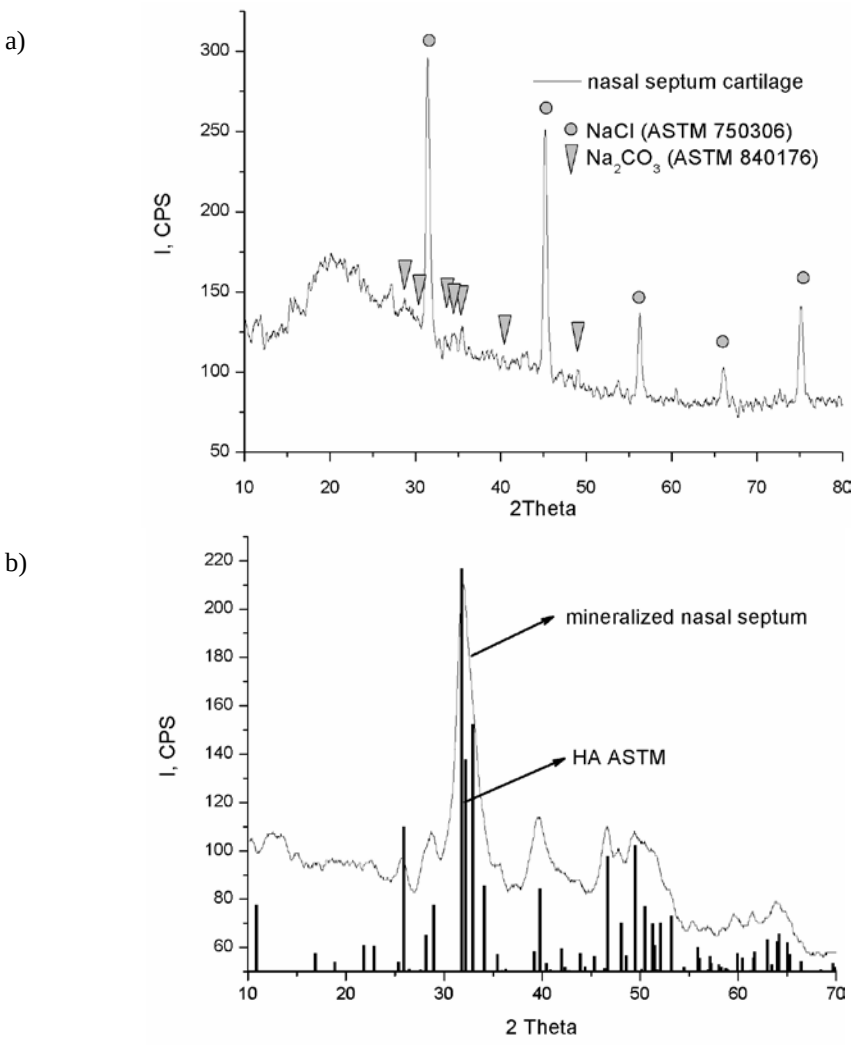

Fig. 1. XRD pattern of a) nasal septum cartilage and b) mineralized nasal septum cartilage

The XRD pattern of the mineralized cartilage (Fig. 1b) reveals the specific peaks of HA which can be distinguished clearly by overlapping the diffraction pattern of the mineralized cartilage with the crystalline HA (ASTM 745066). What is worth to note is that in the case of mineralized cartilage the sodium chloride peaks are not revealed perhaps because of the low quantity of this in comparison with HA. Based on Scherrer equation the size of the main HA crystallites is up to 6nm. The size of the obtained HA is well smaller than the size of the HA obtained in similar conditions but, using collagen matrices which reach up to 20nm.

By comparing the XRD pattern recorded for the nasal septum cartilage with that recorded for mineralized cartilage the nasal septum cartilage has resulted to have a good affinity for mineralization.

3.2. Fourier transform infrared spectroscopy

Infrared spectroscopy (Figure 2) was used to characterize the cartilage and the mineralized cartilage, especially for their organic components. The finger-print area of the nasal

septum cartilage is rich in absorption band and these peaks are overlapped. The main differences between the two spectra are: after the mineralization the phosphate peaks appeared, and some collagen peaks were shifted due to the interactions between the collagen and HA. The HA particles are deposited on the nasal cartilage due to the strong electrostatic interactions between Ca^{2+} cations from HA and carboxylate groups from collagen. Because of such interactions, the COO^- peak is shifted from 1744 (from nasal cartilage) to 1638 cm^{-1} (for mineralized nasal septum). As compared to COLL/HA composite materials obtained by collagen (type I) fiber mineralization, the shifts are more marked [23]. Such result can be explained based on the more complex composition of the cartilage versus the employed collagen fibers.

Based on the literature data [24, 25], the mineralization of the nasal septum cartilage by SBF immersion technique has resulted in a poorly crystalline, immature and non-stoichiometric hydroxyapatite which also contains a small amount of calcium carbonate (attribution made based on the absorption band at 871cm^{-1}).

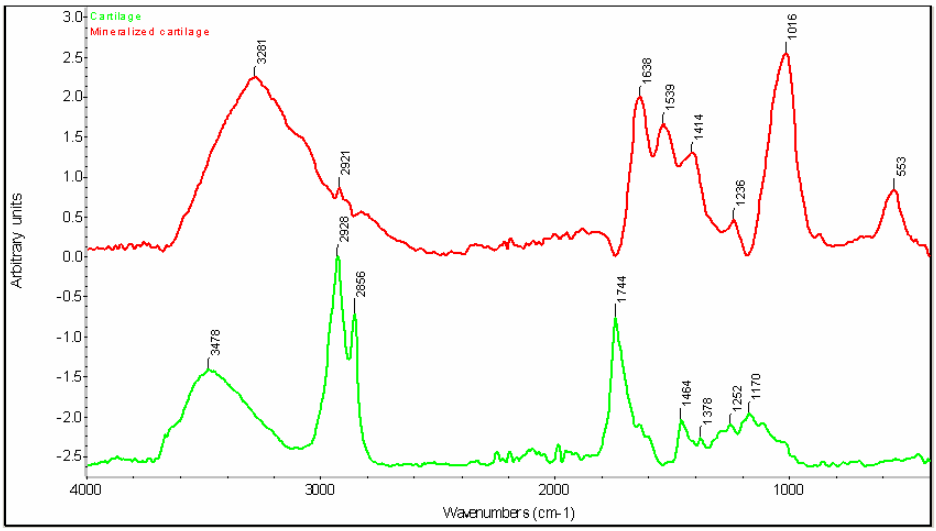

Fig. 2. FTIR spectra of mineralized and unmineralized nasal septum cartilage

3.3. Scanning electron microscopy

Images in Figure 3 (a-c) reveal the microstructure of the nasal septum cartilage at different magnifications and different sections. In the Figure 3a the surface and a side view of the cartilage can be seen. These two areas can be seen better at a higher magnification (4000 x - Figures 3b and c). The cartilage surface is pore–free (compact) due to the particular assembling of the collagenous structures (Figure 3b); in a cross-section view, the V–shape of collagenous fibers can be seen (Figure 3c).

After immersion in SBF, the cartilage was investigated by SEM (Figure 3d-h). Figure 3d and e were taken on the same areas as in Figure 3a and b, respectively, but after mineralization. Figure 3e, f were taken at different magnifications, revealing the mineral particle deposited on the cartilage surface. The Figure 3g and h show a cross sections at different magnifications in order to reveal the HA depositions onto the fibrils and fibers. It is worth to mention that due to the mineralization process, the cross section morphology was changed completely as it can be seen in the pictures from Figure 3c and h, both taken at the same magnification.

Figure 3i shows the SEM image of the composite material obtained by collagen matrix mineralization, SBF immersion. Comparing this image with the previously presented SEM images the two materials can concluded to be very different, especially from the point of view of organic phase morphology and the amount of deposited HA.

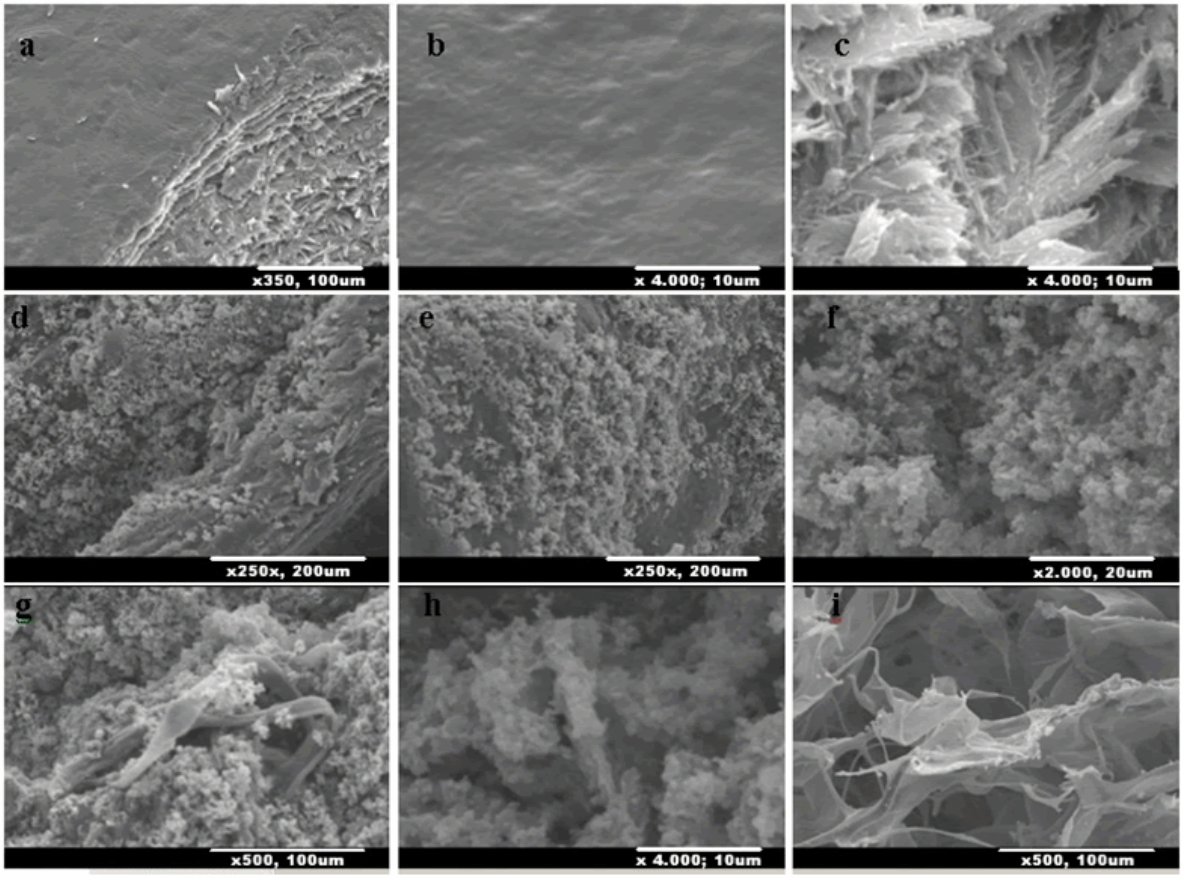

Fig. 3. SEM images of: a–c) human nasal septum cartilage; d–h) mineralized human nasal septum cartilage, by SBF immersion technique; i) mineralized collagen matrix, by SBF immersion technique

3.4. Complex thermal analysis

The complex thermal analysis (Figure 4) was recorded to quantify the content of the three major components from the mineralized cartilage: water, collagen and hydroxyapatite. The thermal analysis also has allowed to differentiate many kinds of water, similar to the case of bones [26].

The three effects corresponding to water loss are: free water evaporation (endothermic effect accompanied by mass loss occurring at ~62^{o}C); chemically bounded water evaporation (endothermic effect accompanied by mass loss occurring at ~202^{o}C and at ~310^{o}C). Summing up, the water level reaches ~57% in the mineralized cartilage. The exothermic peaks at ~398^{o}C and ~484^{o}C correspond to the cartilage damage. The most intense DTA peak is centered at 522^{o}C and corresponds to an exothermic effect due to the burning of the denatured organic matter in the mineralized cartilage, such a process being accompanied by a mass loss. Cartilage denaturation occurred at ~108^{o}C, such a process being accompanied by an endothermic effect and no mass changes. The water evaporation and organic component burning can be considered to be finished at 580^{o}C and based on this observation the HA content can be estimated. The organic content in the mineralized cartilage is ~20% while the mineral phase (mainly HA) is ~ 13%.

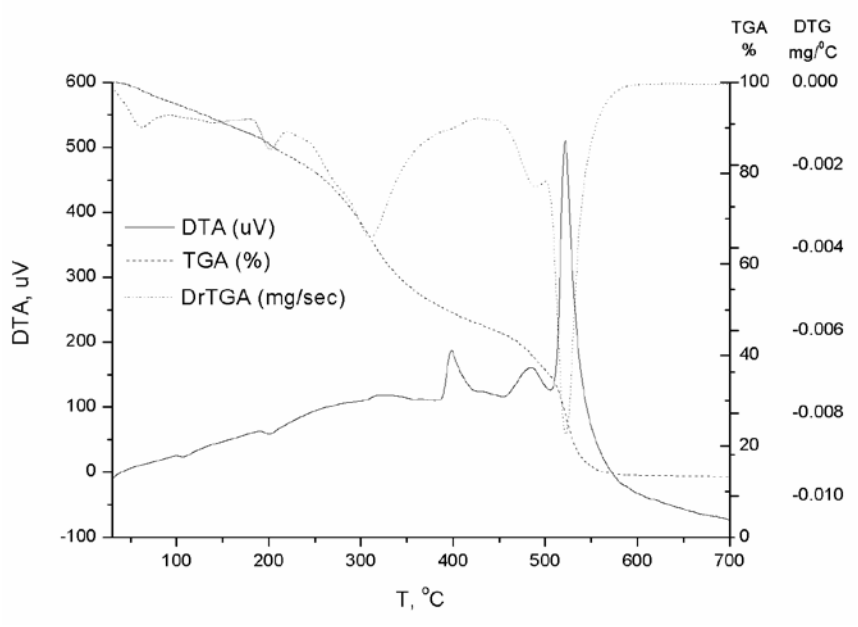

Fig. 4. Thermal behavior of the mineralized nasal septum cartilage

5. Conclusions

Human nasal septum cartilage was obtained by surgical intervention while the mineralized cartilage by SBF immersion technique. It can conclude that human nasal septum cartilage is susceptible to mineralization. The mineralization process can be revealed clearly by XRD, FTIR and SEM while the quantification can be achieved by TG. The mineralization process occurs on the cartilage surface but also in the bulk.

The interaction between cartilage and HA was revealed by the shift of the carboxilate peak from 1744 to 1638 cm^{-1}, meaning that these interactions are very strong. The size of the crystallite estimated by Sherrer equation indicate that the size of the main HA crystallite obtained by nasal septum cartilage mineralization is much more smaller than that obtained by collagen matrices mineralization perhaps due to the increasing of the number of nucleation centers due to the presence of adhesion molecules from the cartilages.

Based on the literature data [27, 28], the use of natural collagenous material (such as cartilage) together with a biommimetic mineralization process (such as the SBF immersion technique) increases the rate of obtaining materials with improved properties (both mechanical and biocompatibility).

Perhaps to the special microstructure of the cartilage, the resulting hybrid composite material has shown a microstructure similar to that of the compact bones. Based on the cartilage microstructure and mechanical properties we expect to improve the mechanical properties of such materials by mineralization.

From a quantitative point of view, the organic: inorganic ratio has reached up to 6:4. In the future we aim at improving the method by increasing the mineralization degree and reducing the mineralization time.

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