New approaches in layer by layer synthesis of collagen/hydroxyapatite composite materials

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Abstract: The aim of this work was to increase our understanding of collagen (COLL)/ hydroxyapatite (HA) composite materials; more specifically, we focused on the study of the influence of the precursor concentrations over the final content of deposited HA. We found that the increase of the precursor concentrations led to better mineralization (on the basis of the content of deposited mineral phase). Regardless of the precursor concentrations, the content of the deposited amount was found to increase with the increase of the number of deposited layers. Quantification of the mineral phase amount was achieved by gravimetric determination. Based on the determined deposition equation the number of layers can be easily determined in order to obtain composite materials with desired content of mineral phase.

Keywords: Bone graft • Collagen • Hydroxyapatite • Morphology • Layer by layer

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1. Introduction

The primary challenges that biomaterials researchers face is the development of new synthetic materials using the tools assured by nanotechnologies and the improvement of existing methods in order to obtain better materials with enhanced biomaterial-host body interactions [1,2].

Many synthetic biomaterials were tested as bone graft materials but the most promising of them seems to be the collagen (COLL)/ hydroxyapatite (HA) (nano) composite materials because these are the most similar to natural bones [1].

In addition to many other domains, intense research is taking place in the field of (nano)medicine to finally obtain nanomaterials that can be used in implant applications and which, if introduced in the human body, will be accepted and receive a positive response from its side – to simulate the human body as much as possible and to serve it [2].

The minerals based on calcium phosphates are the most commonly employed materials in implantology [3] because they are the primary constituents of hard tissues, have superior biocompatibility, and have a higher capacity to directly incorporate into the bone tissue by resorption, (e.g. β-TCP) or by allowing to the new bone tissue to grow inside their porous structure (e.g. HA). This is the reason why calcium phosphates became important materials for bone and dental implants in the last two decades [4-10].

The other main component of bones is type I collagen. Collagen occurs in many places throughout the body. Type I collagen occurs at the level of skin, tendon, vascular ligature, internal organs, and bone. So far, 29 types of collagen have been identified and described in the literature [11,12]. It is important to note that over 90% of the collagen from the body is of type I, II, III, and IV. It can be seen that, depending on the nature and composition of the other components, type I collagen leads to different materials with unique forms and functions, from bone, tendon and cartilage to blood vessels, the cornea, and skin [13,14].

Many types of “layer by layer” (LbL) methods are described in the literature [15-17]. A new method used for obtaining COLL/HA composites is the unconventional “layer by layer” method that implies the immersion of the collagen matrix in a suspension of Ca(OH)₂ followed by the immersion into a NaH₂PO₄ solution. Previous results proved the following: the deposition of the first layers of

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HA is significantly influenced by the collagen matrix; if the collagen matrix is crosslinked with glutaraldehyde, the structure of the matrix does not modify during the process of deposition and the porosity will be reduced along with the number of layers until the saturation point and; following the saturation of the pores, HA will be deposited only on the mineralized surface of the collagen [17].

Some of the advantages of this method, compared to other methods of deposition, are the simplicity, versatility and the possibility to control the quantity of deposited material. The quantity of deposition agent can be controlled by the number of layers and by the quantity of deposited material on each layer during the deposition process. For each layer, the quantity of deposited material can be monitored by means of the following parameters: concentration, deposition time, pH and ionic strength [18].

The main goal of this work is to study the influence of the precursor concentrations on the mineralization process realized by LbL technique on the basis of qualitative and quantitative aspects.

2. Experimental Procedure

The collagen matrices were obtained at the National Research & Development Institute for Textiles and Leather, Collagen Department starting from collagen gel (M.W.=300 kDa) by crosslinking with glutaraldehyde. Calf hide was used in the preparation of collagen gel, through a special chemical-enzymatic process, and purified by dialysis against water [12].

The mineralization process took place directly on the collagen matrix starting from Ca(OH)\(_2\) suspension and NaH\(_2\)PO\(_4\) solution (p.a. Fluka), as schematically shown in Fig. 1. The concentration and volume of the Ca(OH)\(_2\) suspension and NaH\(_2\)PO\(_4\) solution are presented in Table 1. All Ca(OH)\(_2\) suspensions and NaH\(_2\)PO\(_4\) solutions corresponding to C1, C2 and C3 were obtained by using the same amount of Ca(OH)\(_2\) and NaH\(_2\)PO\(_4\).

The mineralization process was performed in two steps. First, the collagen matrices (weighing between 0.0240 and 0.0245 g) were immersed into the corresponding, magnetically stirred Ca(OH)\(_2\) suspension (Table 1) for 30 minutes. Second, after dipping in the Ca(OH)\(_2\) suspension, the matrices were immersed into the corresponding phosphate solution (Table 1) for 10 minutes in order for HA to precipitate. Between the two immersions, the matrices were left for 5 minutes to allow excess water to drain.

The mineralization process by "layer by layer" technique was repeated for each of the 6 collagen matrices. The obtained samples were characterized by scanning electron microscopy (SEM) to visualize the morphological changes versus the number of layers while the HA content evolution was continuously monitored by gravimetric analyses. Also, the samples were qualitatively analyzed by X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and SEM. Each sample was air-dried before characterization.

XRD analysis was performed using a Shimadzu XRD 6000 diffractometer at room temperature. In all cases the Cu K\(_\alpha\) radiation from a Cu X-ray tube was used. The samples were scanned in the Bragg angle, 2\(\theta\) range of 10 – 87\(^\circ\) at a scan rate of 2\(\text{° min}^{-1}\). All samples were ground to fine powders before analysis.

SEM images were obtained with a HITACHI S2600N with an EDAX probe. All samples were covered with a silver layer prior to imaging.

IR spectroscopic measurements were performed using a Vertex 70 (Brucker) spectrometer provided with a diamond crystal based ATR module. The spectra were recorded over the wavenumber range of 400 – 4000 cm\(^{-1}\) with a resolution of 2 cm\(^{-1}\) for collagen matrix as well as COL/H/A composites.

Differential thermal analysis (DTA) coupled with thermogravimetric analysis (TGA) were performed in an air atmosphere with a Shimadzu DTG-TA-50H at a heating rate of 10\(^\circ\)C.

3. Results and Discussion

The composite materials were characterized using both quantitative and qualitative methods of analysis.

3.1. Scanning electron microscopy

Fig. 2 presents the characteristic SEM images recorded for representative samples of the three sets of composite materials obtained by LbL.

It was found that the mineral phase increases as the number of deposited layers increases, and also

| Table 1. Characteristics of precursor suspensions/solutions for C1-C3 |
|---------------------------------------------------------------|
| **Concentration of Ca(OH)\(_2\) suspension, g L\(^{-1}\)**       | C1  | C2  | C3  |
|---------------------------------------------------------------|
|                                                                 | 0.859| 1.718| 3.436|
| **Volume of Ca(OH)\(_2\) suspension, L**                       |     |     |     |
|                                                                 | 0.5 | 0.25 | 0.125|
| **Concentration of NaH\(_2\)PO\(_4\) solution, g L\(^{-1}\)** |     |     |     |
|                                                                 | 1.1168| 2.2336| 4.4672|
| **Volume of NaH\(_2\)PO\(_4\) solution, L**                    |     |     |     |
|                                                                 | 0.5 | 0.25 | 0.125|
increases as the precursor concentrations increase (Fig. 2). For instance, if the number of deposited layers was less than four, in the case of C1, HA agglomerates could not be identified at 1000 or 500x (Figs. 2a, 2b). These agglomerates became visible at these magnifications only after five successive deposited layers (Figs. 2c, 2d). As the concentration of the HA precursors was increased, these agglomerates became visible at lower number of successive layers, for instance, in the cases of C2 and C3 these agglomerates were well defined even at four deposited layers (Figs. 2e, 2f and 2i, 2j).

In the case of C3, it was found that the deposition of seven successive layers of HA resulted in composite materials with well mineralized collagenous matrix (Figs. 2k, 2l).

### 3.2. X-Ray diffraction

XRD patterns of the COLL/HA composite samples were recorded only for qualitative assessments. Regardless of which sample of the three sets was analysed by XRD, the pattern looks similar to that from Fig. 3, in which the intensity of the main HA peaks were found to increase as the number of layers increased. Based on these results, it can be stated that in all cases, the deposited mineral phase contains HA as main component (identifying based on the ASTM 74-5066).

### 3.3. Fourier transform infrared spectroscopy – FTIR

The main absorption bands of collagen are: 1450, 1550, 1650 cm⁻¹ and those of the HA are: 565, 603, 1032, 1423, 1450 cm⁻¹ [19]. The broad peak from 3000-3600 cm⁻¹ corresponds to the hydroxyl associated from water, collagen and hydroxyapatite.

Infrared spectroscopy is used for the qualitative evaluation of the mineralization process based on the increase of the relative intensity of the main phosphate peak (1020 cm⁻¹). In Fig. 4 the IR spectrum of pure collagen and the spectra of the samples C1-4 and C1-7 are presented. It can be seen that due to the mineralization the intensity of the main phosphate peak increases with the increasing number of deposited layers.

### 3.4. Gravimetry

Gravimetrical measurements were used in order to quantify the amount of deposited HA (Fig. 5). The amount of deposited HA was determined in two different ways; in the first case, the amount of deposited HA was determined based on the collagen matrices weight before and after mineralization while, in the second case the content of collagen and HA was determined based on the mass loss at 600 °C. Regardless of the quantification methods used the results were similar.
Figure 2. Characteristic SEM images recorded for some COLL/HA composite materials obtained by LbL, at different HA precursors’s concentration and 1000 and respectively 500x magnification (arrows indicate HA agglomerates).
Figure 2. Characteristic SEM images recorded for some COLL/HA composite materials obtained by LbL, at different HA precursor’s concentration and 1000 and respectively 500x magnification (arrows indicate HA agglomerates).

Figure 3. XRD pattern of C3-7 COLL/HA composite materials.
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Analysing the three curves corresponding to the three HA precursor concentrations it can be stated that, in all cases, the amount HA increases with the number of layers.

It is also noteworthy that the quantity of the HA deposited in the first layer is strongly influenced by the precursor concentrations, the content of deposited HA being proportional with the precursor concentrations. The rate of deposition (which is proportional with the coefficient of the number of layers) is almost the same for the first two sets and slightly increases for the C3.

4. Conclusions

In this work we have accomplished the mineralization of collagenous matrices by a “layer by layer” method, and have performed an assessment of the influence of precursor concentrations on the amount of deposited HA.

The synthesis of COLL/HA composite materials with various levels of resulting mineral phase content is very important in order to obtain bone grafts compatible with different bones.

**Figure 4.** FTIR spectra of the composite collagen matrix and composite materials obtained by immersion of collagen matrices at different concentrations

**Figure 5.** HA content evaluation with the number of layers, determined gravimetrically
The linearity of the deposition is very important because it permits the determination of the number of necessary layers which have to be deposited in order to obtain the desired content of HA. For instance, in the synthesis of the COLL/HA composite materials with 50% HA, the number of layers was 22 for C1; 19 for C2 and 13 for C3.

Our results demonstrate that the LbL method can be used for the reproducible synthesis of COLL/HA composite materials, with desired mineral phase content. Moreover, future studies will involve testing the possibility to apply the LbL method to the COLL/HA composite materials instead of pure collagen matrices, with the precursor solutions modified to contain additional components which may improve the properties of the resulting matrices.

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