

Mimicking the morphology of long bone

Research Article

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Abstract: The aim of this work was to mimic the stratified structure of the median region of long bones. Starting from this desideratum, more COLL/HA composite materials with different morphology were synthesized and characterized, each of these materials mimicking one layer of long bone (endo- and periosteum, compact and cancellous bony tissue). Stratified bone grafts were obtained by assembling these layers; the obtained grafts were similar to the median region of long bones. Even though, natural bones have a more complex microstructure, this is a pioneering work since for the first time a stratified COLL/HA composite material similar to bone was produced.

Keywords: Microstructure • Porosity • Electron microscopy • Assembly
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1. Introduction

Bone is an amazing composite material mainly composed of hydroxyapatite, collagen and water [1]. The mechanical and physico-chemical properties of bones are dependent on composition and especially microstructural characteristics [2-4]. It is well known that compact bone have superior mechanical properties than spongy bone but lower Ca^{2+} , Mg^{2+} and PO_4^{3-} exchange rate.

Long bones have special microstructure, the median zone of these types of bones contain spongy microstructure inside and compact microstructure on the outside region. The transition from the spongy to the compact region is slow, both regions being outside delimited by the medullar canal and muscular tissue attached to the bone by membranes [5-7].

Due to increasing requirement of bone grafts many materials with both compositional and microstructural improvements were synthesized and tested [8-12]. Among other types of bones, some long bones are frequently exposed to fracture [13]. Collagen/hydroxyapatite composite materials seem to be the most suitable materials for bone grafts especially due to the compositional similarity. Unfortunately, in order

to mimic natural bones, still some difficulties have to be resolved, especially those associated with the micro and ultrastructure of these materials [7] because the morphology is one of the most important factors which induces differentiation of mechanical properties of bones and grafts [14].

In the last few years, some very important advances in the field of uniaxially oriented COLL/HA composite materials have been reported [15-18], the synthesis of compact bone substitutes being conditioned by the possibility of obtaining oriented microstructures. From a chronological point of view, the first orientation method involves the use of rotating high magnetic field (10T), the orientation occurring due to the different magnetic susceptibility on the three axes ($\chi_{a,b} < \chi_c$) [15]. The second method of orientation involves the use of a pulsating electric field (0.93 V cm^{-1}), the orientation occurring due to the interaction of electric field with the bipolar structure of collagen molecules, fibrils and fibres [16]. High degree of orientation was also obtained by self-assembly, the orientation being induced by the continuous restructuring of the wet, uncross-linked COLL/HA composite materials at certain conditions [17]. Morphological control has also been recently described, COLL/HA composite materials

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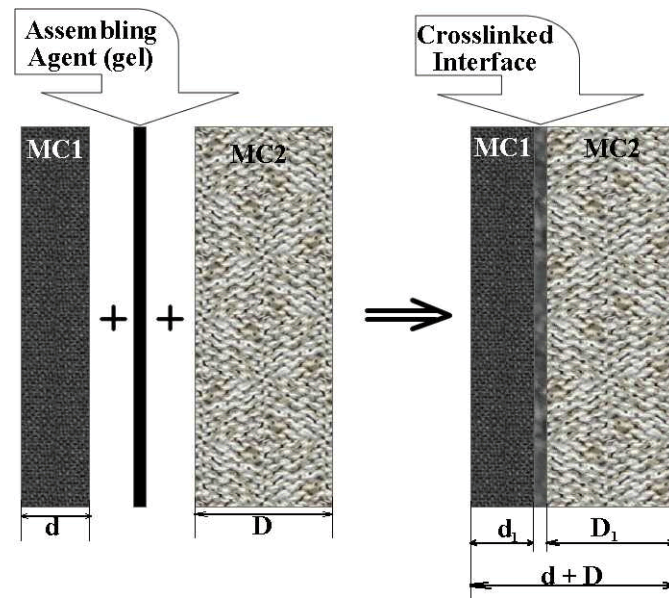


Figure 1. Schematic presentation of the assembly process of two composite layers, with different ceramic properties.

with desired ceramic properties have been produced by combined drying (controlled air drying followed by freeze drying) [18]. Even if orientation methods are now available, the synthesis of complex architectures such as epiphyseal region of long bones (where the orientation of the osteon-like structures follows the lines of force) is not yet possible.

Starting from the need to mimic the stratified morphology of long bones and the possibility to obtain COLL/HA composite materials with both compact and spongy morphology we synthesized potential bone grafts of the median region of long bones. At this moment, a simplified bone graft material which would mimic the four main regions of long bones were obtained starting from the individual analogues: periost, endost, compact and spongy bony tissue.

2. Experimental procedure

Type I bovine collagen (MW=300 000Da) obtained through a special chemical-enzymatic process was used [18-20]. Hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$) was obtained *in situ*, in the presence of collagen gel (1.6%). As precursors, calcium hydroxide ($\text{Ca}(\text{OH})_2$ p.a., Fluka) and sodium dihydrogen phosphate (NaH_2PO_4 p.a., Fluka) were used. The cross-linking of the collagen was done using 1% glutaraldehyde (Sigma-Aldrich) obtained by dilution with water.

Collagen/hydroxyapatite composite materials were obtained under similar conditions as described previously [18]. Briefly, the collagen gel is mineralized in two successive stages. In the first stage, the collagen

gel is treated with the corresponding amount of $\text{Ca}(\text{OH})_2$ suspension, drop wise, and let to interact over 24 h. In the second stage, the stoichiometric quantity of NaH_2PO_4 solution was added and the pH set to 9. The quantities of collagen gel (1.6%) and HA precursors lead to COLL/HA composite materials with COLL:HA ratio of 1:4 for both porous and compact materials while the two films used as periosteum and respectively endosteum analogue consist of two collagen films [21]. In order to obtain composite materials with different microstructural characteristics, combined air drying process was used [18]. The thickness of these four components was set in order to mimic the ratio observed in natural bone: both membranes were ~ 0.1 cm, the analogue of the compact region was ~ 1 cm while the analogue of the porous region was larger than ~ 2 cm. In the case of the two layered materials, COLL/HA composite materials films were assembled in order to prove the versatility of assembling different films with different morphology and thickness.

The assembling process is described in brief in Fig. 1. The two COLL/HA composite matrices, with different microstructural characteristics, are treated with mineralized collagen gel and glutaraldehyde 1%, assembled and then freeze dried. In the same way, the two layers corresponding to the endosteum and periosteum were assembled and the median region of the long bone graft was obtained.

After drying, the composite (layered) materials were analysed from the point of view of morphology and structural aspects by X-ray Diffraction (XRD), Scanning Electron Microscopy (SEM) and some of the ceramic properties (density, porosity and

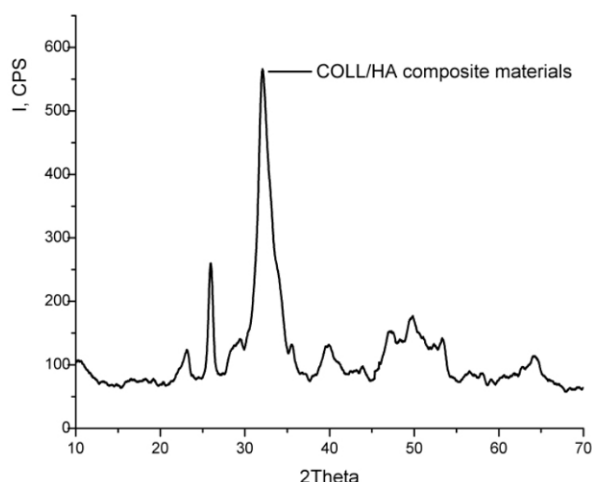


Figure 2. XRD pattern of COLL/HA composite material.

Table 1. Ceramic properties of some COLL/HA composite materials with different morphology.

Sample	Ceramic properties		
	Density (g/cm ³)	Porosity (%)	Absorption of xylene (%)
MC 1	0.2429	78.48	75.62
MC 2	0.4456	57.63	55.39
MC 3	0.5920	51.25	49.85
MC 4	1.1012	43.12	41.56
MC 5	2.0125	19.99	15.34

xylene absorption) were determined by Arthur method.

X-ray diffraction analysis was recorded on a Shimadzu XRD 6000 diffractometer at room temperature, using Cu K α radiation. The samples were scanned in the Bragg angle, 2θ range of 10–70, with a scanning rate of 2 deg min⁻¹.

SEM images were recorded on a HITACHI S2600N electron microscope on the composite samples used for assembling as well as on the assembled bone grafts. Prior to analysis all samples were sputter coated with a thin layer of silver.

The density, the porosity and xylene absorption were determined using the Arthur method, based on the samples weight measured in air, before and after immersion in xylene as well as the samples weight measured by dipping in xylene (using the “pan straddle” system) [18].

3. Results and discussion

XRD is used to set off the mineralization process of collagen gel. The XRD spectrum (Fig. 2) of any of

the composite layers used for assembling reveals the presence of the HA, the differences being insignificant because only HA gives diffraction peaks.

Ceramic properties of the synthesized composite materials are very important especially because the assembling does not affect the bulk properties of these layers only affect the interface properties. Composite materials with different ceramic properties that were obtained for this special purpose are presented in Table 1. Obviously, the use of more porous external layers will induce quicker cell adherence and consequently better osteointegration. The less porous layers will provide higher mechanical strength.

Scanning electron microscopy confirms the asymmetric nature of the assembled materials and reveals a uniform, defect free interface which proves very good assembling of the two components (Fig. 3). It is worth to mention that the morphology of the two components is not altered by the assembly method, morphology changes take place only at the interface. Three different kinds of double layered COLL/HA composite materials were obtained, following the assembly of the two layers. The first double-layered material was obtained by assembling two layers with different ceramic properties (Fig. 3a). The other two materials were obtained by assembling two layers with different ceramic properties and by varying the degree of orientation of the compact (Figs. 3b, 3d) or porous (Fig. 3c) layer. The mineralized collagen gel, used for assembling, penetrates unequally into the two layers, which is obvious in the case of the porous layer (clearly visible in Fig. 3b).

The median zone of long bones was similarly obtained by assembling four successive layers, each of which looked similar to a layer of natural long bone: the exterior layer of bone called periosteum consists of a thin low mineralized collagen layer, the second layer is compact bony tissue; the third layer is spongy bony tissue while, the interior layer, called endosteum, is compositionally and functionally similar to periosteum [5] (Fig. 4a). It has to be noted that the four layered bone analogue is a simplified model of the median zone of natural long bone.

The SEM images of synthesized median zone of long bone graft allow for analysis of the four layers as well as the three interfaces. Due to the thickness of the four-layered materials, the detector does not facilitate analysis of whole materials, even at low magnification, so only two images were recorded. Fig. 4b allows the visualization of the external region of the bone graft (including the external layer similar to periosteum-A, the compact region-B and partially the spongy region of this material). Fig. 4c allows the visualization of the internal

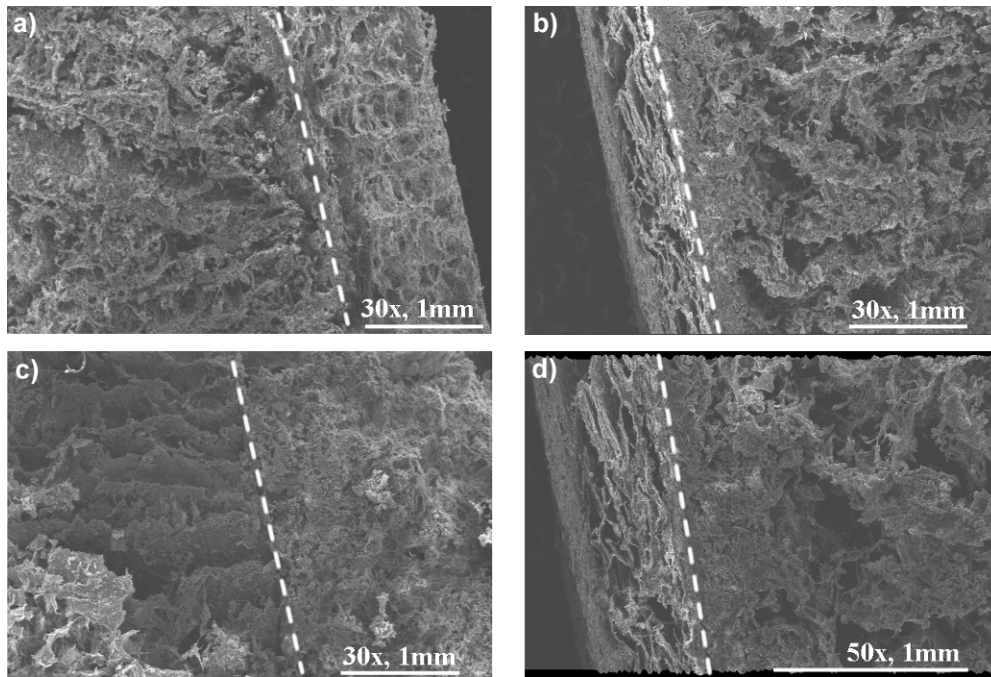


Figure 3. The SEM images of asymmetric, double-layered potential bone grafts obtained by assembling the two COLL/HA layers with different ceramic properties.

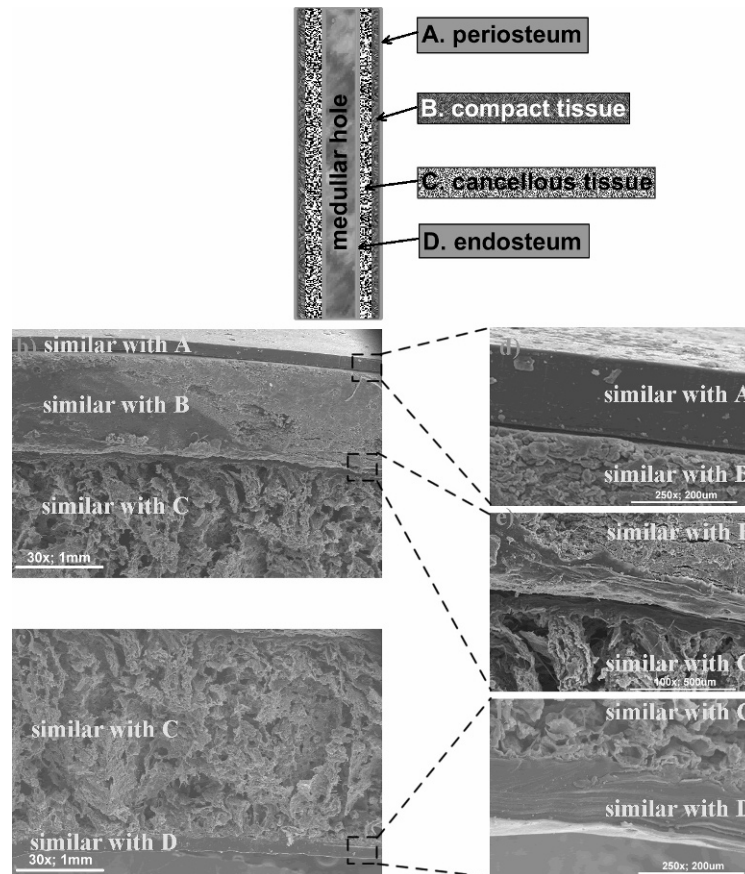


Figure 4. a) Schematic representation of median zone of natural long bones; b, c) SEM images of synthesized median zone of the potential long bone graft, magnification 30 $\times$ and d-f) SEM images of the three interfaces.

region of the bone graft (including the spongy region-C of this material and the internal layer corresponding to the endosteum-D). Figs. 4d-4f presents the three interfaces of the bone graft material, at 100 and respectively 250× magnifications. The two interfaces, which bound the porous layer, are practically defect-free. The interface between the periosteum analogue and compact layer had some defects as a result of the sample preparation procedure (prior to SEM examination which involved mechanical sectioning of the sample).

The heterogeneous nature of the material assures a better reproduction of natural bone together with the possibility that some layers could act as drug delivery system. Based on data from literature a lot of drug delivery systems are known and applied in the field of bone regeneration [22,23].

4. Conclusion

This paper presents a pioneering work, on an easy way to synthesize multilayered analogue of the median zone of long bone graft. The presented method involves the synthesis of COLL/HA composite layers with

different morphologies followed by their assembling. The assembling is done using mineralized collagen gel (similar to the composition of the assembled layers) and cross-linked with glutaraldehyde. Even though, currently the complete synthesis of the long bone is not possible due to difficulties in preparation of more complex oriented structures (femoral head, for instance), the presented method can be easily used for preparation of partial grafts of the median zone of long bones.

Due to the layered nature each layer can have a specific role (other than the above mentioned roles related to the four main components of the long bones), for instance, the porous layer can act as a drug release reservoir while the other layers may limit the delivery rate.

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