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ORIGINAL PAPER

Histochemical and Scanning Electron Microscopic Characterization of Tricalcium Phosphate – Collagen Conjugated Sponges

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Abstract

In the last decade, the research on materials for bone regeneration has focused on materials that are degradable and capable of stimulating tissue regeneration. In this context, we assume that a porous composite of tricalcium phosphate (β-TCP) and collagen (COL) offer an interesting alternative, given the wide range of solubility they present and their similarity with bone composition. The collagen-phosphate sponges were obtained by the reaction of β-TCP with type I COL under the physiological conditions. Three variants of COL/β-TCP composites were prepared and characterized in light microscopy by von Kossa staining and by scanning electron microscopy. Our electron microscopical results showed that all the sponge-like composites of COL/β-TCP presented a microporous structure with small aggregates of β-TCP non-homogeneous disposed on the COL fibrous bundles. Von Kossa staining revealed a progressive loading of the collagenic material with β-TCP deposits, together with the decrease of the COL:β-TCP combining ratio.

Keywords: collagen, tricalcium phosphate, sponges, composites, light microscopy, scanning electron microscopy

Introduction

Bone is one of the few tissues in the adult human body whose ability to regenerate spontaneously has been recognized. In cases of large bone defects, where bone is not expected to regenerate spontaneously, it must be induced the formation of new bone, using tissue (graft) substitutes made of metals, ceramics, polymers or composite materials [1]. The primary goal is restoration of form and function [2], ideally by having the defect populated with material closely resembling the original bone prior to damage. Restoring function by bone regeneration represents a fundamentally different approach. By this strategy, not only can the re-establishment of physical function be achieved, but also full physiological function may be realized.

Bone represents a complex system, made of various cell types embedded in a matrix consisting of collagen (COL) and calcium phosphate crystals. This organization lends bone high resistence against compression, tension, bending and torsional forces. The high porosity of bone is an optimal compromise between load-bearing capacity and mass. Bone undergoes constant remodelling by osteoclasts and deposition of new bone material by osteoblasts [3]. Intervention becomes necessary when this delicate balance is disturbed.

Various materials, either naturally derived or synthetic, have been tested as bone substitutes [4, 5, 6, 7]. Among the acellular systems are, for example, materials derived from natural bone: COL and mineral phase of human or animal bone [8]. Recently, tricalcium phosphate (TCP) has been attracting attention due to its good biodegradability and biocompatibility [9, 10, 11, 12].

The aim of the present paper was to obtain a new type of material for bone regeneration based on COL/β-TCP porous composites and to investigate their structural characteristics by light and scanning electron microscopy.

Materials and Methods

1. Preparation and characterization of COL/β-TCP conjugated sponges. **Type I COL** was isolated from bovine tendon by pepsin treatment, using dilute acetic acid. The following characteristics of the collagen solution were determined: Hyp and hexosamine content [13], total protein content [14] and molecular weight [13]. **β-TCP** was prepared by sintering the stoichiometric mixture of $NH_4H_2PO_4$ and $CaCO_3$ (2:3 molar ratio), at 1100^0C. Ceramic materials (TCP and intermediate compounds) were characterized on a wide range of temperatures, by X-ray diffraction analysis performed on a Zeiss HZG4A2 high-resolution diffractometer. **Porous composites** were prepared by freeze-drying of the COL solution (0.6% w/v) mixed previously with β-TCP (5% w/v) dissolved in saline phosphate buffer, in the presence of HEPES 0.2M and incubated at 35^0C, for 24 h. The COL/β-TCP composite was obtained in three variants: P1 (COL:β-TCP 1:1), P2 (COL:β-TCP 1:2) and P3 (COL:β-TCP 1:4). A control sponge was prepared using COL alone.

2. Scanning electron microscopy (SEM). The obtained composites were analyzed by SEM, using a scanning electron microscope ESEM, XL-30, FEI (Philips). Samples were processed in the 'low vacuum mode' and visualized as cross-sections.

3. Histological examination. COL/β-TCP sponges were fixed in 4% para-formaldehyde buffered with 10mM phosphate (pH 6.9), washed, dehydrated in graded ethanol solutions, cleared in xylene and embedded in paraffin. 10 μm-thick sections were stained with von Kossa technique in which phosphate deposits were stained in black. The sections were then counterstained with 1% Safranin O for collagen which was stained in red.

Results and Discussions

1. *Synthesis of COL/β-TCP composites*

Tissue engineering is a new field of research that ussually couples synthetic materials with natural ones, like cells and/or protein [15-16]. Our study deals with scaffolds composed of synthetic TCP and natural COL. The main reason of this choice is the preponderence of these components in the bone tissue. Another study had used, as scaffold for dental tissue reconstitution, a biomaterial composed of powdered hydroxyapatite (HA) and COL [17].

In the present study, we have used type I COL, extracted from mature bovine tendon by a non-denaturing method, with optimized parameters (pH, temperature, reagent concentration). The obtained collagen solution was thoroughly characterized, by reproducible techniques, and the analytical results are given in **Table 1**. The value for the average molecular weight of collagen is comparable to that of tropocollagen (300,000 Da). This observation evidenced that the native triple helix structure is not affected after the enzymatic procedure used by us.

Table 1. Analytical determinations for the type I collagen from bovine tendon

Determination	Value (g/100g dry substance)*
Total nitrogen content	15.22
Total protein content	88.40
Hydroxiproline content	9.35
Collagen	85.00
Hexosamines	10.80
Average molecular weight	308,000 Da

*Except the value for molecular weight

β-TCP was obtained from calcium carbonate and ammonium phosphate by thermal decomposition. X-ray diffraction analyses during the calcination process showed that, up to 540^0C, the mass loss was generated by the water and ammonia release after the reagents' decomposition. When the temperature rose to 900^0C, the loss was due to the residual water and, prevalent, to CO_2 from calcium carbonate decomposition. The total mass loss was approx. 41% for each mole of resulted β-TCP. In the temperature range of 500^0-800^0C it took place the formation of intermediate compounds, dicalcium phosphate and tetracalcium phosphate, resulting in the stoichiometric compound, β-TCP. The X-ray diffraction patterns (**Figure 1**) showed that the formation of β-TCP started at temperatures higher than 800^0C, when the carbonate was decomposed. Above 1100^0C there were polymorphous transformations; at approx. 1180^0C, the β form changed to the less stable α form and above 1450^0C, there was a α-α' transformation.

COL/β-TCP conjugated sponges were prepared by the reaction of β-TCP, having hydration activity, with type I COL giving a gel, in a short time, under physiological conditions. Three types of COL/β-TCP sponges were synthesized, in a weight ratio of 1:1 (sample P_1), 1:2 (sample P_2) and 1:4 (sample P_3), respectively.

2. Structural analysis of the obtained sponges

Macroscopically, the three types of sponges were similar, with a microporous structure. The macroscopic appearance is a direct consequence of the intrinsic fibrillary structure of the non-denatured protein [18].

All three sponges had a honeycomb structure with pores of several microns in diameter. In higher magnification of the matrices, it were observed β-TCP deposits, non-homogeneous disposed on COL fibrous bundles. The amount of β-TCP deposits on the COL surface was higher for the P_3 sample than in P_1 and P_2 samples (**Figures 2A-D**).

Light microscopy studies revealed a progressive loading of the collagenic material with β-TCP deposits, together with the decrease of the COL:β-TCP combining ratio (**Figures 3A-C**). Thus, the P_1 sample, with the lowest proportion of β-TCP, presented the smallest deposits of β-TCP, while in the P_3 sample, which had the highest proportion of β-TCP, the collagenous network was intense loaded with β-TCP (**Figure 3C**).

In **conclusion**, our data demonstrated that the association of COL with β-TCP determined stable composites, which prevented the β-TCP dispersion in medium, resulting in an easily moulded biomaterial. These biocomposites presented a microporous structure with small aggregates of β-TCP non-homogeneous disposed on the COL fibrous bundles and they could be used as acellular scaffolds for bone regeneration.

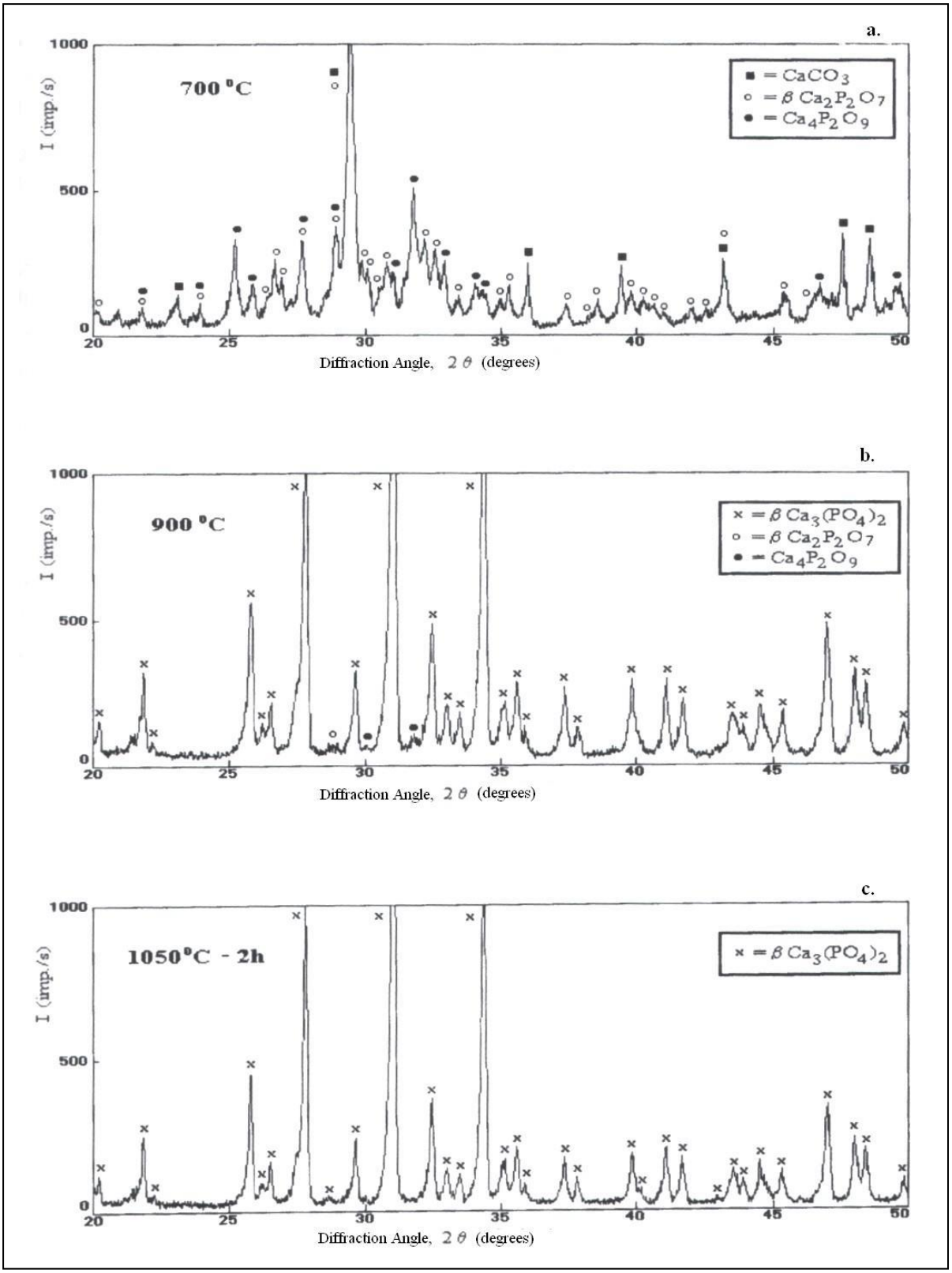


Fig. 1. Kinetics of TCP formation analyzed by X-ray diffraction.
(a) formation of intermediate compounds, dicalcium phosphate (°) and tetracalcium phosphate (•); (b) presence of intermediate compounds and β-TCP; (c) formation of β-TCP.

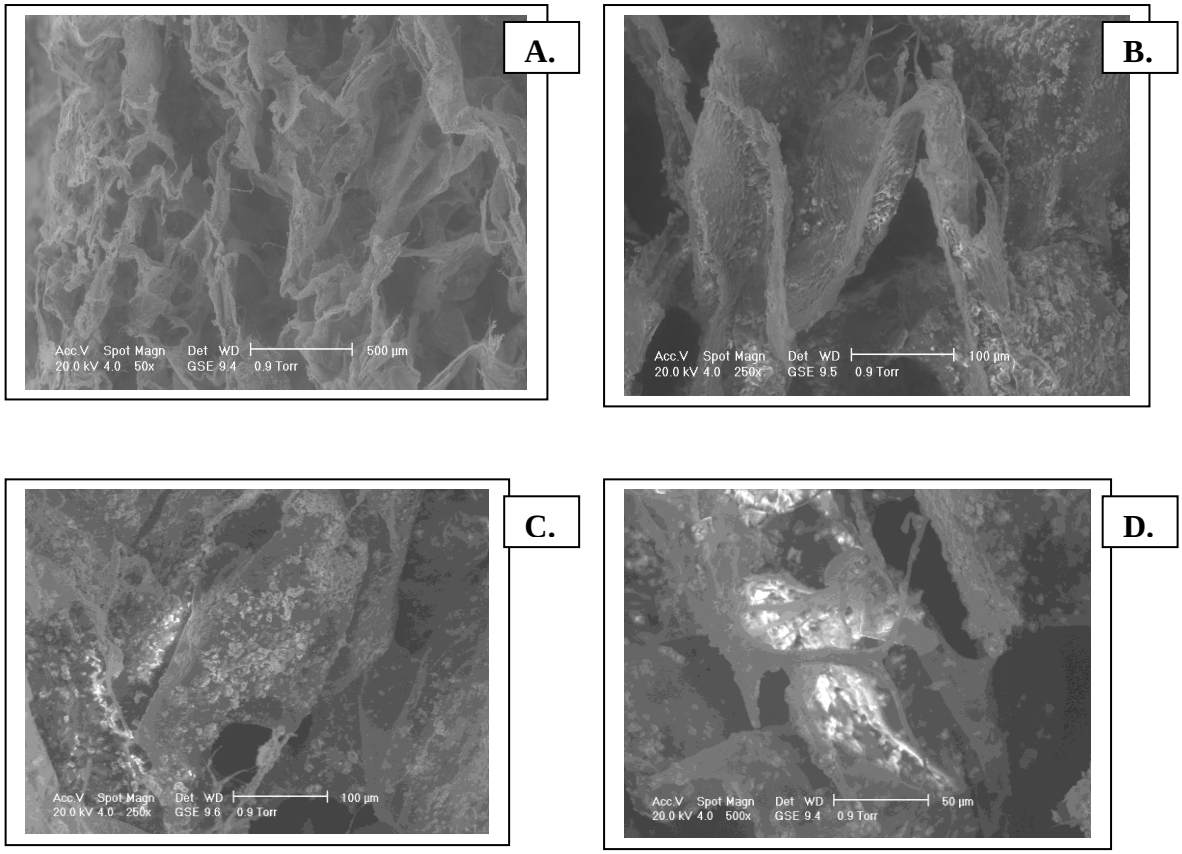
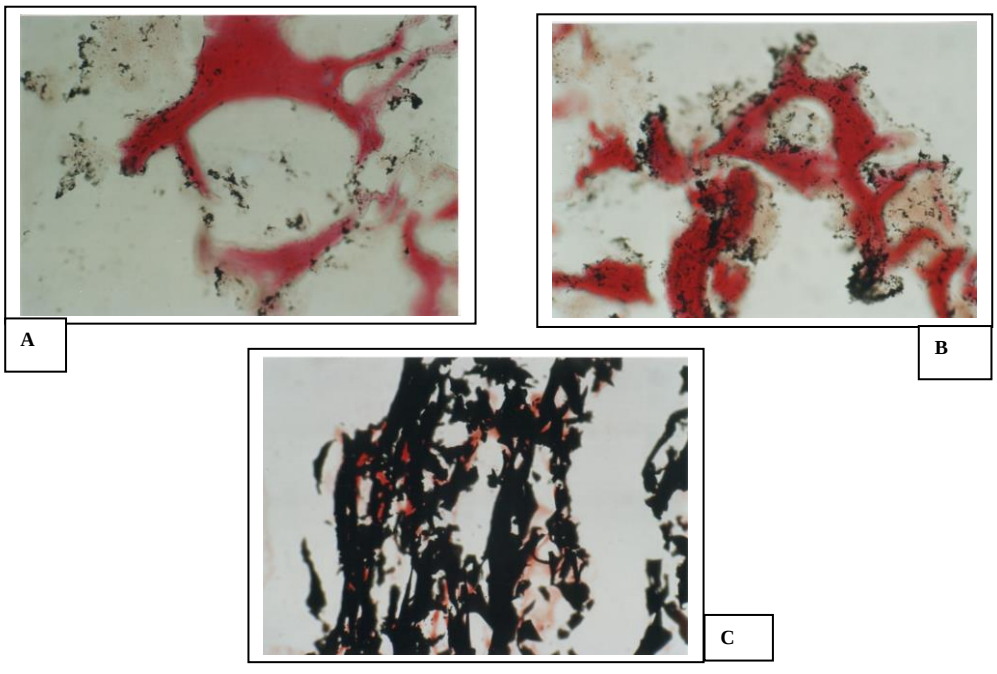


Fig. 2. SEM images of COL sponge (A) and COL-β-TCP biocomposites (B, C, D).
B – P1 sample; C – P2 sample; D – P3 sample

Fig. 3. Tricalcium phosphate detection in COL-TCP sponges by von Kossa method.
A – P1 sample; B - P2 sample; C – P3 sample



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