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THERMAL BEHAVIOR OF IN VITRO MINERALIZED ANIONIC COLLAGEN MATRICES

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In the present study porcine skin and bovine pericardium were used as a source of type I collagen. Both were submitted to an alkaline treatment and mineralized by the alternate soaking method. Thermal stability and extent of mineralization have been investigated using DSC and TG. After alkaline hydrolysis there is a decrease in thermal stability but mineralization stabilizes collagen structure. Thermogravimetric data have shown that the amount of hydroxyapatite present in bovine pericardium matrix (45%) was greater than on porcine skin matrix (20%). Presence of hydroxyapatite was confirmed by EDX.

Keywords: *collagen, hydroxyapatite, mineralization*

Introduction

Bone tissue engineering is a research area with clinical applications in bone replacement on orthopedic defects, reducing the risks and expenses of using autografts or allografts. The mineralization of collagen in vitro [1, 2] is of great interest for the understanding of the mechanisms underlying the mineralization in vivo as well as for the synthesis of improved bone grafts. The substitution of bone is a frequent issue in medicine. Since collagen and calcium phosphate are the major constituents of human bone, bone implant materials consisting of these substances have been under study [3]. Collagen is an important biomaterial in medical applications due to its special characteristics, such as biodegradability and weak antigenicity [4]. Thus collagen, has found many biomedical applications such as implants scaffolds for artificial organs [5], drug carrier systems [6] and bone tissue reconstruction [7].

Several methods of mineralization of collagen scaffolds are described in literature [8, 9] showing the formation of the calcium phosphates such as hydroxyapatite. However the quantification of this mineralization was not reported. The aim of this work is the thermal characterization (DSC, TG) and quantification of mineralization content for anionic collagen matrices obtained from porcine skin (PS) and bovine pericardium (BP) by alkaline hydrolysis. The alkaline treatment increase the negative charges into the collagen triple-helix structure by hydrolysis of carboxyamide groups of asparagine (Asn) and glutamine (Gln) [10] at pH physiological, in order to develop anionic collagen and acellular materials [11]. Mineralization was done by alternate soaking method [12]. TG determines the inorganic phase content in the matrices. Mineralized type I collagen also has a potential to be utilized as a biomimetic material to augment or repair calcified tissues that may be impaired, diseased, or defective.

Experimental

Anionic collagen matrices preparation (PS96 and BP96)

Porcine ventral skin (PS) was acquired from slaughterhouse. PS was mechanically cleaned up and rinsed in a 0.9% saline solution and deionized water. BP (bovine pericardium) was obtained from animals aged 30–60 months and supplied by Braile Biomédica, SA, São José do Rio Preto, SP, cleaned and defatted as described for the routine of cardiac bioprothesis manufacture.

PS and BP were submitted to the alkaline hydrolysis, as a habitual procedure in our laboratory [10]. Briefly, PS and BP tissues were treated at 25°C for 96 h in alkaline solution containing 6% (*v*/*v*) dimethyl sulfoxide, salts of alkaline and alkaline earth metals. The resulting material was placed in a solution of sulfates and chlorides of Na^+, K^+ and Ca^{2+} for a period of 6 h and the excess of residual salts was removed by successive washings with 3% boric acid, 0.3% EDTA at pH 11 and deionized water, equilibrated in 0.01 mol L^{-1} H_3PO_4 solution for 24 h. After this procedure the matrices were washed with distilled water, lyophilized and stocked at 25°C. Hydrolyzed matrices obtained after that treatment were denominated PS96 and BP96.

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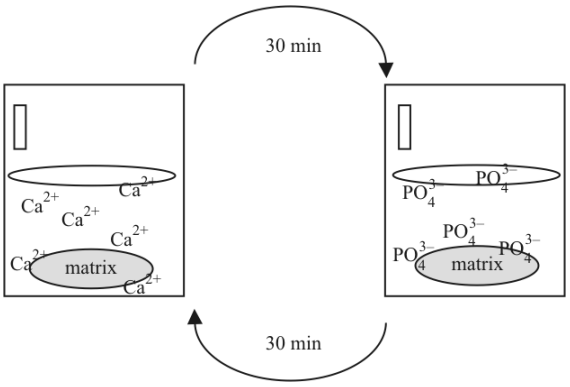

Fig. 1 Alternated soaking method

In vitro mineralization

The mineralization process (Fig. 1) was carried out as follows:

- PS96 and BP96 soaked in 60 mL of 0.2 mol L^{-1} $CaCl_2$ buffered with 0.05 mol L^{-1} Tris buffer (pH 7.4) at 37°C for 30 min.
- PS96 and BP96 taken out of the Ca^{2+} solution and were rinsed with deionized water.
- PS96 and BP96 were subsequently soaked in the 0.12 mol L^{-1} Na_2HPO_4 solution buffered with 0.05 mol L^{-1} Tris buffer (pH 9.0) at 37°C for 30 min.
- PS96 and BP96 were taken out of the PO_4^{3-} solution and were rinsed with deionized water.

The alternate soaking cycle was repeated 6× to obtain the mineralized matrices and then the matrices were rinsed with water, frozen and lyophilized in the Edwards Model Freeze Dryer Modulyo, Edwards High Vacuum International (West Sussex, UK). Mineralized matrices were denominated PS96C and BP96C.

Methods

Differential scanning calorimetry (DSC)

Calorimetric measurements were performed using a DSC-2010, Thermal analyzer (TA Instruments, New Castle, DE, USA) with temperature calibration with indium standard. Prior to the test collagen matrices were immersed in phosphate buffer 0.13 mol L^{-1} (pH 7.4) for 24 h. Liquid in excess was removed by compression using an absorbent paper, and the sample was hermetically sealed in an aluminium pan. Heating was carried out at 10°C min^{-1} in the temperature range 5–120°C, using ice water as the cooling medium, in synthetic air flow (80 mL min^{-1}) and an empty pan was used as the reference. Sample mass is in the range 9–10 mg. Denaturation temperature (T_d) was determined as the inflection point value of the corresponding endothermic effect.

Thermogravimetric analysis (TG)

Thermogravimetric analysis was carried out using a TGA 2050, TA Instruments (New Castle, DE, USA). Heating was performed in a platinum crucible in synthetic air flow (90 mL min^{-1}) at a rate of 10°C min^{-1} up to 800°C. The sample mass was in the range of 9–10 mg.

Scanning electronic microscopy (SEM)

Samples of about 1 cm^2 were coated with a thin layer of gold-palladium of 20 nm. The specimens were examined with a LEO 440, LEO Electron Microscopy Ltd. (Cambridge, England) with an accelerating voltage of 20 keV.

Energy dispersive X-ray analysis (EDX)

Ca/P ratio was obtained by EDX equipment LEO 440, LEO Electron Microscopy Ltd. (Cambridge, England), with an Oxford detector Mod. 7060, Oxford Instruments Inc. (Concord, USA) with 133 eV resolution and samples previously coated with carbon at a distance of 20 mm. Standards: $CaCO_3$, quartz, GaP and Wollas ($CaSiO_3$).

Results and discussion

Thermal behavior of matrices was studied by differential scanning calorimetric (DSC) and thermogravimetric analysis (TG). DSC provides a sensitive means of understanding the thermal denaturation events when collagen is heated [13]. Denaturation can be defined as a transition from the triple helix to a randomly coiled form due to many factors such as heat, acid, alcohol and other agents. When collagen is submitted to high temperatures, intramolecular crosslinks are broken, and the protein undergoes a transition from a highly organized crystalline structure to a random, gel-like state that is denominated gelatin [14].

Figure 2 shows DSC curves of collagen scaffolds before and after mineralization. DSC curves of the matrices show a thermal transition referring to the denaturation (T_d) of the collagen.

Untreated porcine skin (PS) and bovine pericardium (BP) had a T_d of 67°C. Matrices submitted to the alkaline hydrolysis (matrices PS96 and BP96) had a significant decrease in thermal stability (Table 1). The reduction in thermal stability of anionic collagen matrices of about 24°C may result from the cleavage of natural cross-links promoted by the alkaline condition used to hydrolyse Asn and Gln amides to carboxyl groups [11]. Although there is a decrease of denaturation temperature (T_d), the triple helix structure was preserved

since denatured collagen is devoid of thermal transition in the temperature interval studied [13].

It was observed also an increase of the T_d (4°C) due to the mineralization process, indicating that the salt deposition increases the stability of the triple helix. There are experimental evidences that mineralization increases the thermal stability of the collagen molecules and temperature of transition depends on degree of mineralization [15]. Thermogravimetric analysis was carried out for all matrices (Fig. 3) and the inorganic phase content of the samples were determined as the residue at 750°C.

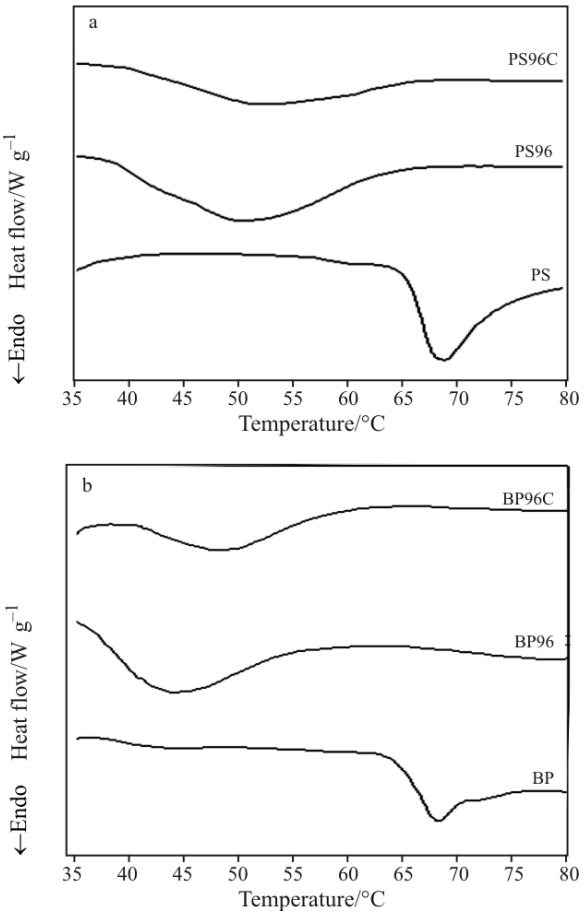

Fig. 2 DSC curves for a – PS, PS96 and PS96C, b – BP, BP96 and BP96C (10°C min^{-1}, synthetic air flow)

Table 1 Denaturation temperature (T_d) of collagen matrices as measured by DSC

Matrices	T_d/°C
PS	67
PS96	42
PS96C	47
BP	67
BP96	39
BP96C	43

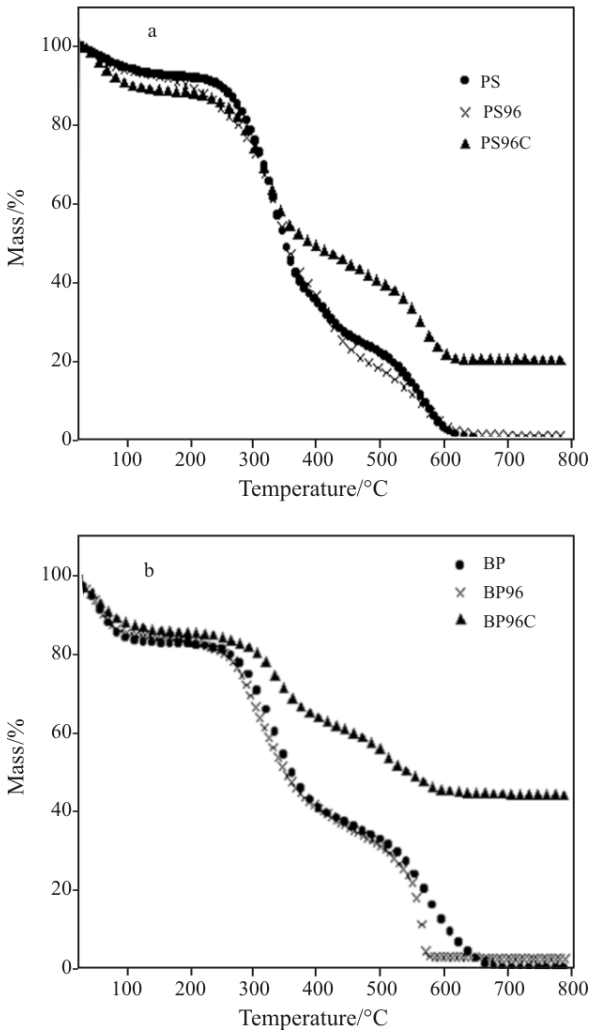

Fig. 3 TG curves for a – PS, PS96 and PS96C, b – BP, BP96 and BP96C (10°C min^{-1}, synthetic air flow)

Thermal behavior is characterized by three stages of mass loss. The first one between 25 and 200°C is associated with release of water content of samples, the second around 200–400°C is due to the degradation of the structure of the collagen molecule and the third that occurs in the range 400–650°C is related to the combustion of the residual organic components. Residues were obtained at 750°C (Table 2) and allowed to determine an inorganic material content as reported in Table 2, since hydroxyapatite (product more desirable during mineralization) is stable in the studied temperature interval [16]. The residue of non-mineralized matrices PS, PS96, BP is less than 1%, and is about 2% for BP96. The mineralized matrices BP96C and PS96C showed a residue of 45.8 and 20.5%, respectively, attributed to the presence of calcium phosphate salts. These results showed that mineralization process is most effective for matrices obtained from bovine pericardium.

Table 2 Mass loss (%) of collagen matrices as measured by TG

Matrices	Mass loss/%			Residue/% at 750°C	Collagen degradation temperature/°C
	25–200°C	200–400°C	400–650°C		
PS	7.7	57.3	34.6	0.4	264.5
PS96	12.2	49.4	37.5	0.9	245.1
PS96C	11.9	38.9	28.7	20.5	264.8
BP	15.1	42.9	41.3	0.7	264.5
BP96	13.6	41.6	42.8	2.0	256.7
BP96C	12.5	22.3	19.4	45.8	280.3

In the second stage, due to collagen degradation, it can be observed a decrease in the thermal stability after alkaline hydrolysis and an increase when occur mineralization (Table 2), suggesting that mineralization stabilized collagen structure.

Photomicrographs of surfaces of the matrices PS96, PS96C, BP96 e BP96C are shown in Fig. 4. It is clearly observed the presence of precipitated crystallites in mineralized matrices. Crystals show morphology of needle-like shape that are grouped in spherical aggregates. However PS96C morphology shows aggregates of relatively larger size (Figs 4b and 4d). The crystal morphology observed in the surface of both matrices suggest hydroxyapatite formation, since very similar pictures were reported earlier for HA crystallized on metal surfaces [2, 17].

In order to verify the Ca/P ratio EDX analysis was carried out (Fig. 5). The EDX performed on the samples revealed that the main elements of the mineral were carbon (0.2 keV), oxygen (0.5 keV), calcium (3.7 and 4.0 keV), and phosphorus (2.0 keV). The Ca/P molar ratio were 1.66±0.12 and 1.65±0.02

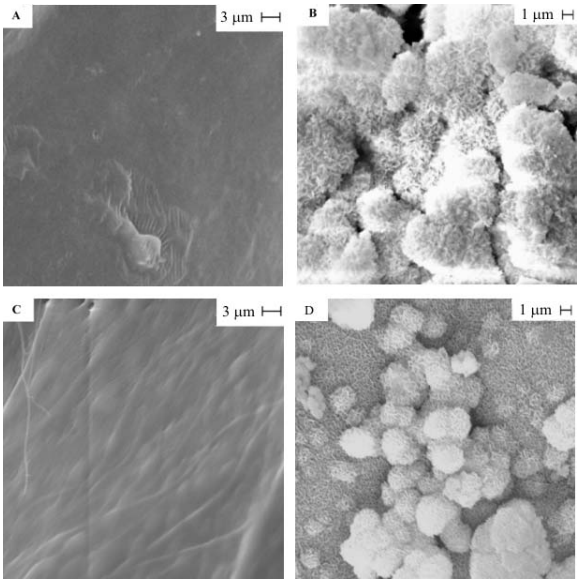

Fig. 4 SEM image of surface matrices: A – PS96, B – PS96C, C – BP96 and D – BP96C

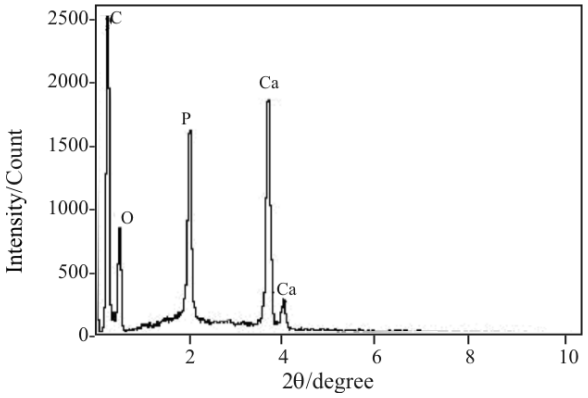

Fig. 5 Energy dispersive X-ray (EDX) for the mineralized matrix (electron beam, 20 keV)

for matrices PS96C and BP96C, respectively which is a little below the theoretical value of 1.67 for hydroxyapatite.

Conclusions

TG and SEM data reveal that both matrices were successfully mineralized, however with differentiated quantities. Mineral content, as determined by thermogravimetry showed that bovine pericardium matrix was more mineralized than porcine skin. Thermogravimetric and calorimetric data showed that mineralization stabilizes collagen structure. EDX showed a Ca/P ratio of 1.65, which is a little below the theoretical value of 1.67 for hydroxyapatite.

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