COLLAGEN-INDOMETHACIN-HYDROXYAPATITE SPONGIOUS FORMS FOR BONE RECONSTRUCTION TREATMENT

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The aim of this study was to prepare and characterize collagen spongious forms for bone tissue engineering. The ideal scaffold has almost the same structural and biological functions as a naturally occurring extracellular matrix in terms of physical structure and chemical composition Having about 30% collagen and 70% hydroxyapatite, to mimic bone composition, the obtained matrices were characterized by water up-take, enzymatic degradation, scanning electron microscopy and indomethacin release from supports. The results showed that the composites based on collagen and hydroxyapatite absorbed less water than the ones with indomethacin. The degradation process showed that indomethacin inhibits collagenase solution, degradation taking place in more than one week. The pore sizes are proper for bone tissue engineering and the release of drug is fast in first 4 hours and slowly next hours. The results showed us that the combination between collagen, indomethacin and hydroxyapatite is a promising spongious form, slowly degraded in vitro, with specific absorbance properties, with proper pore sizes and porosity and which can release the drug rapidly, ensuring a diminution of the inflammation and of the associated pain; this formulation could be successfully used in bone regeneration.

Keywords: collagen, indomethacin, hydroxyapatite.

INTRODUCTION

The scaffolds for bone tissue engineering should possess several properties such as biocompatibility, mechanical similarities with the target tissue and high porosity for cell adhesion, proliferation, and tissue development. The ideal scaffold has almost the same structural and biological functions as a naturally occurring extracellular matrix in terms of physical structure and chemical composition (Mederle *et al*., 2016).

Collagen is an important constituent of the natural extracellular matrix (ECM) and matrices made from it are used as scaffolds for tissue engineering as well as systems for drug delivery. Collagen is also one of the main components of the bone and that's why most of the materials designed for bone grafting and repair are based on collagen and hydroxyapatite. Collagen matrices have porous structures which influence hydrophilicity, drug diffusion through the network, the degradation properties and interaction with and within cells (Ficai *et al*., 2010).

Hydroxyapatite is a naturally occurring substance that is found in teeth, bones, and tendons. The hydroxyapatite is the one main inorganic component of bone and it has a variety of uses in bone applications such as fillers and replacements due to its excellent biocompatibility, osteoconductivity and biointegration (Rusu *et al*., 2015; Popa *et al*., 2016).

Indomethacin (IND) is a nonsteroidal anti-inflammatory drug commonly used as a prescription medication to reduce fever, for pain, stiffness, and swelling from inflammation. It works by inhibiting the production of prostaglandins, molecules known to cause these symptoms and ease the pain and discomfort (Cabezas, 2012).

The aim of this work was to obtain collagen-hydroxyapatite-indomethacin matrices, uncross and cross-linked with glutaraldehyde with variable pore sizes for hard tissue engineering.

MATERIALS AND METHODS

Collagen Matrices Preparation

Collagen (Col) gel was obtained from bovine skin using the protocol that has been previously described (Albu, 2009). An amount of 0.2% indomethacin (IND), and 70% hydroxyapatite (HA) was embedded under mechanical stirring into collagen gel having the concentration 1% and pH 7.2-7.4. The gels with compositions according to Table 1 were then cross-linked with 0.025% glutaraldehyde (GA) and put in Petri dishes of 3 cm diameter. The collagen gels were freeze-dried using the Delta LSC 2-24 (Martin Christ, Germania) freeze-dryer for 48 hours and the corresponding matrices were obtained. Compositions and codes of the sample are given in Table 1.

Table 1. Compositions and name of collagen gels

Code of gels	Code of matrices	Col*, %	HA**, %	GA**, %	IND*, %
Col	B1	1	0	0.025	0
Col-IND	B2	1	0	0.025	0.2
Col-HA	B3	1	70	0.025	0
Col-HA-IND	B4	1	70	0.025	0.2

* reported to collagen gel volume

** reported to collagen dry substance

Water Up-take Capacity and Enzymatic Degradation

The water uptake capacity and enzymatic degradation were performed using the protocol as we previously described (Albu, 2012) on the obtained matrices with compositions according with Table 1.

Scanning Electron Microscopy

The scanning electron microscopy (SEM) images were registered using FEI Quanta 200. Matrix morphology and pore shape and sizes were determined with SEM.

In vitro Release of Indomethacin

The *in vitro* kinetics release of indomethacin from the obtained matrices was determined using a “sandwich” device adapted to a paddle dissolution equipment (Essa Dissolver), as detailed in our previous studies (Ghica *et al*., 2014). The amount of indomethacin released of different time intervals in the receiving medium (phosphate buffer pH 7.4) was spectrophotometrically evaluated at λ=268 nm (Perkin-Elmer UV-

Vis Spectrophotometer), using the calibration curve ($A_{1\%}^{1cm} = 454$, $R^2 = 0.9990$) (Figure 1).

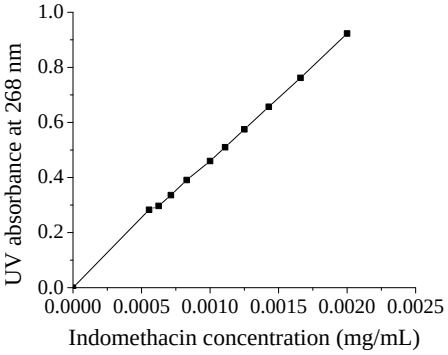

Figure 1. Calibration curve of indomethacin in phosphate buffer (pH 7.4)

The experimental kinetic data were analyzed with the Power law model (eq. 2).

$$\frac{m_t}{m_\infty} = k \cdot t^n \qquad (2)$$

where, m_t/m_∞represents the fraction of drug released at time t, k - the kinetic constant, n - the release exponent characteristic for the drug release mechanism.

RESULTS AND DISCUSSIONS

After lyophilization, sponges based on collagen, hydroxyapatite and indomethacin were obtained. Water absorption capacity for the B1÷B4 matrices is showed in Figure 2.

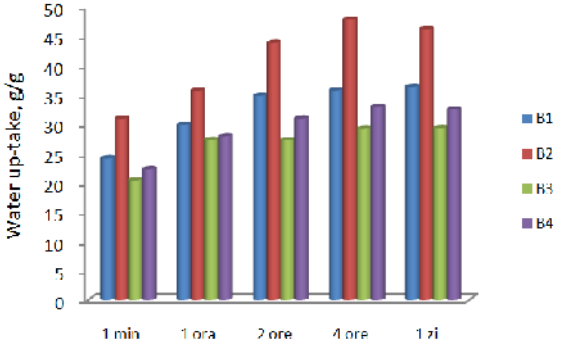

Figure 2. Water absorption of the matrices

The prepared matrices absorbed between 29 and 46% water. The samples are very stable in water, the values at 4 hours being similar with values for 24 hours. Although

the indomethacin is an hydrophobic drug, the sample which contain it are more hydrophilic then the one without: after one day B1 absorbed 36 g/g and B2 - 46 g/g water and B3 absorbed 29 g/g and B4 – 32 g/g. The samples with hydroxyapatite content absorbed less about of water then the other without.

Enzymatic degradation of matrices are presented in Figures 3 and 4.

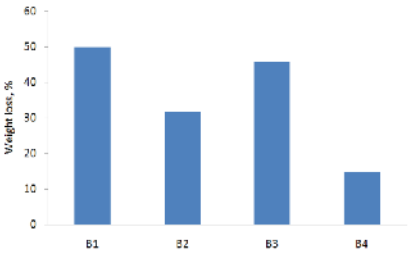

Figure 3. Matrices degradation during 24 hours

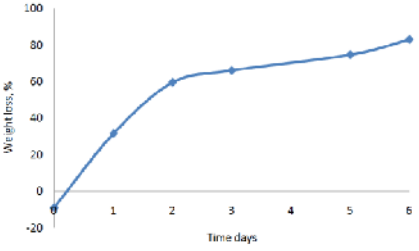

Figure 4. Kinetics of degradation for sample B2, based on Coll and IND

During 24 hours the matrices collagen reference (B1) degraded about 50% and collagen with hydroxyapatite 45%. The indomethacin inhibits collagenase and matrices B2 and B4 degraded in 24 hours 31.6% and 14.7% respectively. This demonstrate that the combination between collagen, indomethacin and hydroxyapatite provide the best stability in simulated environment. The results are in correlation with water up-take.

SEM microscopy shows characteristic pore structure that has a large variation in average pore diameter, for each matrix (Figures 5a, b and c).

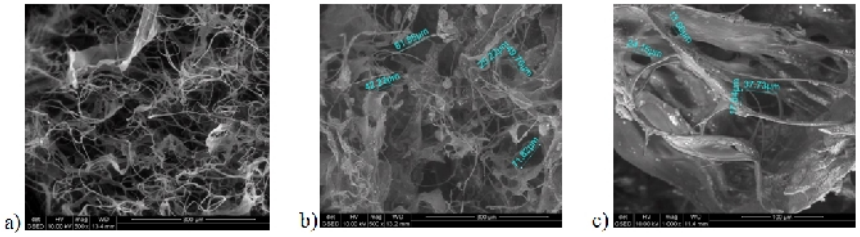

Figure 5. SEM images of matrices: a) B1 (x500); b) B4 (x500); c) B4 (x1000) – transversal section

Figure 5 presents some example of SEM images of matrices. The collagen reference sample, B1 (figure 5a) presents specific interconnected pores of about 100 – 200 µm. In Figures 5b and c we can clear see the hydroxyapatite deposited on collagen fibrils and pores with smaller sizes (between 13 and 81 µm) which give more compact structure for sample B4. These results are in accordance with water up-take and enzymatic degradation.

The influence of the formulation factors on indomethacin release patterns from collagen matrices B2 and B4 is illustrated in Figure 6.

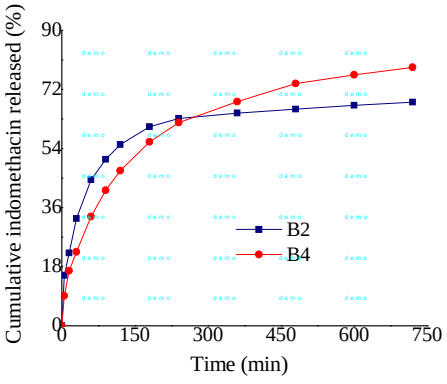

Figure 6. Cumulative release profiles of indomethacin from collagen matrices as a function of time

As can be noticed from Figure 6, the release of indomethacin from collagen matrices tested during 12 hours of experiments recorded two stages. For both samples, in the first two hours, the drug was rapidly release ensuring a diminution of the inflammation and of the associated pain, followed by a gradually and slower drug release during the next hours.

For the matrix with hydroxyapatite, the release of indomethacin was slower in the first 4 hours in comparison with the sample without HA, but after 12 hours of experiments the drug released percentage for this matrix was higher (about 1.16 times) (Table 2). It seems that the presence of bioactive ceramic induced an increase of drug released percentage, this results being in accordance with our previous studies (Ghica *et al.*, 2015).

Table 2. Values for correlation coefficients and kinetic parameters specific to Power law model, and drug released percentage after 12 hours

Collagen matrices	Kinetic constant ($1/min^n$)	Release exponent	Correlation coefficient	Released percentage (%)
B2	0.159	0.234	0.9713	68.11
B4	0.078	0.360	0.9902	78.69

Applying the Power law model to the release data, the kinetic parameters were determined (Table 2). The values obtained for the release exponent (smaller than 0.5) indicated an anomalous drug transport mechanism.

CONCLUSIONS

Composites matrices based on collagen, nano-hydroxyapatite and indomethacin as anti-inflammatory were obtained by freeze-drying in spongious forms. They were characterized by water up-take and enzymatic degradation. The results showed that because of compact structures, the composites based on collagen and hydroxyapatite

absorbed less water, meanwhile the indomethacin induces hydrophilicity to matrices. The degradation process showed that collagen reference crosslinked with glutaraldehyde is degraded about 50% in one day, meanwhile, the collagen with indomethacin, after 6 days degraded in proportion of 83%. The pore sizes of samples with collagen are between 100 and 200 μm and the one with collagen, hydroxyapatite and indomethacin has smaller pore sizes, up to 80 μm. For the matrix with hydroxyapatite, the release of indomethacin was slower in the first 4 hours in comparison with the sample without HA, but after 12 hours of experiments the drug released percentage for this matrix was higher (about 1.16 times). The results showed us that the combination between collagen, indomethacin and hydroxyapatite is a promising spongious form, slowly degraded in vitro, with specific absorbance properties, with proper pore sizes and porosity and which can release the drug rapidly, ensuring a diminution of the inflammation and of the associated pain; this formulation could be successfully used in bone regeneration.

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