**DESIGN AND EVALUATION OF
DOXYCYCLINE/COLLAGEN/CHONDROITIN SULFATE DELIVERY
SYSTEMS USED FOR CARTILAGE REGENERATION**

MARIA-MINODORA MARIN^{1,2}, MĂDĂLINA GEORGIANA ALBU KAYA^{2*}, MIHAELA VIOLETA GHICA³, ELENA DĂNILĂ¹, GHEORGHE COARĂ², LĂCRĂMIOARA POPA³, CIPRIAN CHELARU², DURMUŞ ALPASLAN KAYA⁴, VALENTINA ANUȚA³, CRISTINA-ELENA DINU-PÎRVU³, IOAN CRISTESCU⁵

¹*University Politehnica of Bucharest, Faculty of Applied Chemistry and Materials Science, 1-7 Gheorghe Polizu Str., 011061, Bucharest, Romania*

²*National Research and Development Institute for Textile and Leather, Division Leather and Footwear Research Institute, Collagen Department, 93 Ion Minulescu Str., 031215, Bucharest, Romania; albu_mada@yahoo.com*

³*“Carol Davila” University of Medicine and Pharmacy, Faculty of Pharmacy, Physical and Colloidal Chemistry Department, 6 Traian Vuia Str., 020956, Bucharest, Romania,*

⁴*Mustafa Kemal University, Faculty of Agriculture, Department of Field Crops, 31034, Antakya-Hatay, Turkey*

⁵*Carol Davila” University of Medicine and Pharmacy, Faculty of Medicine, Department of Orthopaedy, 8 Bulevardul Eroilor Sanitari, 050474, Bucharest, Romania*

Cartilage damage is difficult to self-heal due to an avascular microenvironment and distinct mechanical properties. These features are a challenge in designing a cartilaginous tissue with repairing effect without producing any local infections. Thus, a biodegradable scaffold in which the drug can be incorporated is preferable. Drug delivery systems based on collagen sponges have progressively become remarkable biomaterials for different medical applications. The aim of this work was to design and characterize some collagen/chondroitin sulfate supports with doxycycline for cartilage tissue regeneration. The doxycycline should prevent the development of potential infections. Collagen, chondroitin sulfate and doxycycline gels were cross-linked with different concentrations of glutaraldehyde and then freeze-dried in order to obtain collagen matrices. The structural characteristics for the new synthesized biomaterials were firstly assessed by infrared spectroscopy (FT-IR), and scaffolds morphology was then evaluated by optical microscopy and water uptake. The enzymatic biodegradation was also performed. Also, the sponges surface properties were quantified through contact angle. The *in vitro* doxycycline kinetics release was performed with a dissolution equipment and the release mechanism was investigated. The obtained results recommend these new scaffolds based on doxycycline/collagen/chondroitin sulfate as a promising approach for the treatment of cartilage problems.

Keywords: collagen, doxycycline, chondroitin sulfate

INTRODUCTION

The cartilage tissue is powerless to self-repair and restore due to the absence of nerves, blood vessels and lymphatic tissues, which makes the treatment more difficult (Huang *et al.*, 2016). These features are a challenge in designing a cartilaginous tissue with repairing effect without producing any local infections. Thus, a biodegradable scaffold in which the drug can be incorporated is preferable (Song *et al.*, 2017; Huey *et al.*, 2012). Collagen, a natural biomaterial, represents the main protein from the human organism: skin, bone, cartilage, organs, blood vessels, ligaments and tendons (Miao *et al.*, 2018). Collagen exhibits excellent properties, such as low antigenicity, gradual biodegradability, good biocompatibility and optimal cell proliferation (Lan *et al.*, 2019). Drug delivery systems based on collagen sponges have progressively become remarkable biomaterials for different medical applications (Vikash *et al.*, 2013).

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Chondroitin sulfate (CS), a fundamental component of extracellular matrix in conjunctive tissue (cartilage, bones etc.), is a glycosaminoglycan which helps the formation of aggrecans in the native cartilage (Bang *et al.*, 2018). Collagen and chondroitin sulfate can be great candidates for the design of cartilage tissue engineering in controlling cellular performances (Zhang *et al.*, 2011; Cao *et al.*, 2008). Doxycycline hyclate (DH) represents a tetracycline antibiotic with a strong broad-spectrum activity against aerobic microorganisms and it is usually used in the treatment of the infections (Raval *et al.*, 2014). Thus, the aim of this work was to design and characterize some collagen/chondroitin sulfate supports with doxycycline, for cartilage tissue regeneration.

MATERIALS AND METHODS

Materials and Preparation of the Scaffolds

The type II collagen was extracted from bovine cartilage using technology currently available at the National Research and Development Institute for Textile and Leather, Division Leather and Footwear Research Institute – Collagen Department. Doxycycline hyclate and chondroitin sulfate were purchased from Sigma-Aldrich, China. Sodium hydroxide and hydrochloric acid were of analytical grade. Type I collagenase obtained from *Clostridium histolyticum* was purchased from Sigma-Aldrich, Germany and glutaraldehyde (GA) from Merck, Germany.

The concentration of each collagen gel was adjusted at 1.5% and 7.4 pH using 1M sodium hydroxide. 0.2% DH and different concentration of CS were added to collagen gel (w/v), and then the collagen gels were cross-linked with 0.0025% GA as Table 1 presents. The samples were coded as follows: M-1÷M-5.

Table 1. Composition of the obtained samples

Sample	Doxy, %	CS, %
M-1	0	0
M-2	0.2	0.05
M-3	0.2	0.10
M-4	0.2	0.15
M-5	0	0.05

FT-IR Analysis

FT-IR spectral measurements were recorded by a Jasco FT-IR 4200 spectrophotometer. All the spectra were recorded at the following parameters: spectral range 4000 - 600 cm⁻¹, resolution 4 cm⁻¹ with 30 acquisitions per each sample.

Optical Microscopy Analysis

The morphology of the designed sponges was carried out using a LEICA optical microscope model S8AP0, with increase power of 20-160x.

Contact Angle Evaluation

The sponges surface wettability was assessed with CAM 101 (KSV Instruments), using the pendant drop dynamic method, as reported in our previous works (Ghica *et al.*, 2013; Popa *et al.*, 2013).

Water Uptake

In order to evaluate the water absorption, the obtained samples were immersed in ultrapure water. At timetabled intervals, the samples were weighed. The experiment was done in triplicate. The water uptake was calculated using the equation:

$$\% \text{ Water uptake} = \frac{W_t - W_d}{W_d} \times 100 \quad (1)$$

where W_d is the weight of the dry samples and W_t is the weight of the swollen sample at immersion time t .

Degradation Studies

Enzymatic degradation of sponges was investigated by measuring the weight loss depending on contact time to collagenase solution. At specific intervals, the swollen scaffolds were weighed. The percentage of scaffold degradation was determined by the following equation:

$$\% \text{ Weight loss} = \frac{W_i - W_t}{W_i} \times 100 \quad (2)$$

where W_i is the initial weight and W_t is the weight after time t .

Drug Release Kinetics Analysis

In vitro drug release from collagen spongy matrices was carried out using a “sandwich” device adapted to a paddle dissolution equipment, as described in our previous studies (Albu *et al.*, 2010). The concentration of doxycycline hyclate released in the medium (phosphate buffer pH 7.4) at different time intervals was monitored by UV spectroscopy and the cumulative released drug percentage was evaluated.

RESULTS AND DISCUSSION

The collagen gels with compositions according to Table 1 were freeze-dried and spongy matrices were obtained and characterized.

From the FT-IR spectra (Figure 1) the characteristic bands from collagen it can be observed: amide A, B, I, II and III for all the samples (Albu, 2011).

Figure 1. FT-IR spectra

Figure 2. Optical microscopy (20X)

There are no significant changes when doxycycline hyclate and different concentration of chondroitin sulfate were added to collagen gel.

The optical results for the obtained samples are present in Figure 2. Optical images have shown for all the samples a highly porous structure with interconnected pores presenting various dimensions and shapes which is beneficial to the cell adhesion and proliferation.

The spongy matrices surface wettability was expressed through contact angle (CA°) value. The drop shape (Figure 3), monitored with a digital camera, was mathematically described by the Young equation (eq. 3):

$$\gamma_{SG} = \gamma_{SL} + \gamma_{LG} \cdot \cos \theta \quad (3)$$

where γ_{SG} is the interfacial tension S/G, γ_{SL} – the interfacial tension S/L, γ_{LG} – the superficial tension L/G and θ – the contact angle.

Figure 3. Images of the drop shape for the spongy matrices and the corresponding contact angle values: a) M-1; b) M-2; c) M-3; d) M-4; e) M-5

As it can be noticed in Figure 3, the contact angle values are smaller than 90°, with values between 55.64 and 89.03°, indicating an adequate hydrophilicity of the sponges surface and consequently a good wetting ability by the biologic fluids at the application site. The CA is strongly influenced by the sponges formulation. Thus, the highest value of CA was recorded for the sample M-1 without drug and chondroitin sulfate, and with a high collagen concentration. The addition of CS (0.05%) in the sponge formulation (M-5) lead to the smallest CA, the decrease being about 1.6 times. It seems that the CS presence produced a more porous structure of the sponge surface allowing a better wetting. On the other hand, for the spongy matrix with the same concentration of CS (0.05%), but with 0.2% doxycycline hyclate (M-2) an increase of CA about 1.25 times was observed. These results are in line with our previous studies which highlighted that doxycycline has a cross-linking effect (Albu *et al.*, 2009), inducing in this way a decrease of sponge surface hydrophilicity. Concerning the sponges M-2÷M4 with the same concentration of biopolymer, drug and cross-linking agent, the increase of CS amount determined an increase of CA, more obvious for higher concentration (0.15%).

The water uptake for the studied samples is presented as kinetics during 24 hours in Figure 4. In Figure 4 it can be observed the water absorption during a 24 hours period for the studied samples. All the samples exhibited a high water uptake, this being in concordance with contact angle studies. The maximum amount of water was absorbed by M-5 sample which presented the best hydrophilicity.

Figure 4. Water uptake during 24 hours for spongy forms

Figure 5. Enzymatic degradation over 48 hours for spongy forms

The degradation results (Figure 5) indicated an excellent biodegradation for all the samples during the 48 h time interval. The samples with slower degradation rate were M-4 and M-1 with approximately 50% and 60% weight loss in 48 hours.

The *in vitro* doxycycline kinetic profiles from spongy matrices were recorded as drug cumulative released percentage as a function of time (Figure 6).

Figure 6. Cumulative release patterns of doxycycline hyclate from collagen spongy matrices as a function of time

The kinetic profiles are similar, with an initial burst release in the first 2 hours, followed by a gradual and prolonged drug delivery over the next 48 hours. The most pronounced rapid release effect was recorded for the M-3 sponge with a medium concentration of chondroitin sulfate (38.37%), followed by the M-2 sponge (28.85%) with the smallest concentration of CS, while the less evident burst effect was reported for the M-4 sponge (19.13%). The cumulative doxycycline hyclate released percentage after 48 h has varied between 55.62 (M-4) and 74.29 (M-2) (Table 2). This long-term antibiotic release provides a local and protective antibacterial effect over a longer period of time necessary for tissue repair.

To set up the drug mass transfer mechanism, the kinetic data were fitted to the Power law model (equation 4 and its particular case, Higuchi model ($n=0.5$), the corresponding correlation coefficients (R) values being listed in Table 2:

$$\frac{m_t}{m_\infty} = k \cdot t^n \quad (4)$$

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.

The highest values for the correlation coefficients were recorded for the Power law model, indicating a non-Fickian mechanism for drug release from collagen spongy matrices, in line with our previous studies (Ghica *et al.*, 2013). The release exponent and kinetic constant values specific to the Power law model are given in Table 2.

Table 2. Correlation coefficients for the Power law and Higuchi models; parameter values specific to the Power law model; percentage of drug released

Spongy matrices	R Higuchi model	R Power Law model	Release exponent, n	Kinetic constant, k (1/min ⁿ)	Drug Released (%)
M-2	0.9402	0.9806	0.31	0.069	74.29
M-3	0.8849	0.9850	0.22	0.133	67.38
M-4	0.9524	0.9801	0.34	0.039	55.62

CONCLUSIONS

All the designed sponges presented adequate goniometric, morphological and biological properties. The drug release patterns presented a biphasic shape, targeted

both to prevent and to control local infection at affected tissue, providing a protective antibacterial effect over the required period to favor long-term healing. The results obtained recommend these new scaffolds based on doxycycline/collagen/chondroitin sulfate as a promising approach for the treatment of cartilage problems.

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