COMPOZITE PE BAZĂ DE COLAGEN/CERAMICĂ BIOACTIVĂ/DOXICICLINĂ PENTRU DEFECTELE OSOASE
COLLAGEN / BIOACTIVE GLASS CERAMIC / DOXYCYCLINE COMPOSITES FOR BONE DEFECTS

MIHAELA VIOLETA GHICA[1], ANTON FICAI[2*] , ŞTEFANIA MARIN[2], MINODORA MARIN[2], ANA-MARIA ENE[2], JENEL MARIAN PĂTRAŞCU[3]

[1]*"Carol Davila" University of Medicine and Pharmacy, Faculty of Pharmacy, Physical and Colloidal Chemistry Department, Traian Vuia Str., no. 6, 020956, Bucharest, Romania*
[2]*University Politehnica of Bucharest, Faculty of Applied Chemistry and Material Science, , 1-7 Polizu Str., 011061 Bucharest, Romania*
[3]*"Victor Babeş" University of Medicine and Pharmacy, Faculty of Medicine, Department of Orthopaedics, Traumatology, Urology and Imaging, P-ța Eftimie Murgu, no. 2, 300041, Timişoara, Romania*

The aim of this study was to develop and characterize some new drug delivery systems based on collagen, bioactive (apatite – β-wollastonite) glass ceramics and doxycycline in order to be used in infected bone defects. The composites with different amount of glass ceramics were prepared as hydrogels by crosslinking and then freeze-dryed in order to obtain porous structures which mimic bone. The hydrogels were characterized by rheological behaviour and the the spongious forms by Fourier transform infrared spectroscopy (FT-IR) and water up-take. The in vitro behaviour was determined by collagenase degradation; the doxycycline release from spongious composite systems was investigated and the kinetic mechanism was determined. The results of this study indicated that the obtained composites are promising biomaterials for treatment and prevention of infected bone.

Scopul acestui studiu a fost dezvoltarea şi caracterizarea unor noi sisteme de cedare a medicamentului pe bază de colagen, sticlă ceramică bioactivă (apatită – β-wollastonită) şi doxiciclină în scopul utilizării în defectele osoase infectate. Compozitele cu cantități diferite de sticlă ceramică au fost preparate sub formă de hidrogeluri prin reticulare şi apoi liofilizate pentru obținerea de structuri poroase care mimează osul. Hidrogelurile au fost caracterizate din punct de vedere reologic, iar formele spongioase prin spectroscopie de infraroşu cu transformată Fourier (FT-IR) şi absorbție de apă. Comportarea in vitro a fost determinată prin degradare în prezența colagenazei; a fost investigată cedarea doxicilinei din sistemele compozite spongioase şi a fost determinat mecanismul cinetic. Rezultatele acestui studiu au arătat că sistemele compozite obținute sunt biomateriale promițătoare pentru tratamentul şi prevenția osului infectat.

Keywords: *collagen, bioactive glass ceramic, composite, drug delivery systems*

1. Introduction

Nowadays millions of patients are suffering from bone defects, about 1 million procedures being annually performed in Europe. The worldwide market of bone replacement material is estimated at 5 billion € and is expected to annually increase with 10% especially to aged population [1,2]. Materials used in bone tissue regeneration have been rapidly developed in recent years in order to replace autografts and allografts. Scaffolds which mimic bone, with optimal architecture for cell migration, interaction and differentiation are the most indicated structures for successful functional tissue-engineered bone regeneration [3-5]. Bones are mostly made up of a composite material which incorporates mineral calcium phosphate, hydroxyapatite and collagen, an elastic protein which provides fracture resistance [6].

Collagen is one of the most used biomaterials due to its excellent biocompatibility, biodegradability and its interaction with the body [7]. A high number of biomaterials have been used in tissue reconstruction research, but collagen-based scaffolds were proven to provide the best results [8].

As mineral part of bone, calcium phosphate salts (apatites), especially hydroxyapatite (HA), have been mostly used as bioactivity component, due to their similarity to natural bone composition [9]. Wollastonite ($CaSiO_3$) is calcium-silicate-based ceramic with excellent bioactivity and degradability, used as a potential material for bone tissue regeneration [10].

An innovative mineral material, PAW1 is an apatite-β wollastonite glass ceramic that has been successfully employed in biological applications [11]. It has been used as a composite matrix with

* Autor corespondent/*Corresponding author,*
E-mail: anton.ficai@upb.ro

type I collagen by Bajali et al. demonstrating that it supports bone cell migration, adhesion, growth and differentiation [12]. *In vitro* experiments have shown that the bioactive material used helped the bone structure repair and had no toxic or allergic reactions. Negreanu et al. also used PAW1 as a composite matrix to treat bone defects. *In vitro* and *in vivo* experiments made on dogs and rabbits have demonstrated that the composite matrix formed by collagen and glass ceramic prompts cells to repair bone tissue [11]. In addition to demonstrating the biocompatibility and capacity of tissue regeneration, PAW1 was also tested by Negreanu et al. [13] in a different shape, namely granules. They proved that PAW1 granules can be used as a bone substitute.

Even many collagen/ceramics biomaterials were studied so far [14-20] as successful materials for bone defects, one of the biggest problems is infection and inflammation of bone tissue [21]. Various antibiotics such as gentamicin [22], cephalexin [23] and vancomicin [24] were succesfully released from calcium sulfate/HA composites [25].

Doxycycline is a broad spectrum tetracycline antibiotic with the ability of inhibiting the matrix metalloproteins activity. It plays major roles in cell behavior such as proliferation, migration, angiogenesis, apoptosis and differentiation. Doxycycline is also known for its inhibitory effect on collagenases, bone resorption and its efficient diffusion in bones [26, 27].

The aim of this study was to develop and characterize the novel three-dimensional (3D) bioactive scaffolds used in bone-tissue regeneration. These 3D systems consist of collagen as release support, doxycycline as drug that prevents infections and PAW1 which provides better osteointegration and regeneration.

2. Materials and methods

2.1. Materials

The type I fibrillar collagen gel having a concentration of 1.72% (w/w) was extracted from calf hide using technology currently available at the Research-Development Textile Leather National Institute Division Leather and Footwear Research Institute – Collagen Department [7]. Doxycycline hyclate (DH) was purchased from Sigma-Aldrich, China and PAW1was provided by SC PONETI SRL in 2006. 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), monobasic potassium phosphate and disodium hydrogen phosphate from Merck, Germany.

2.2. Preparation of collagen hydrogels

The concentration of each collagen gel was adjusted at 1% and 7.3 pH using 1M sodium hydroxide (the pH of the physiological medium). PAW1 and doxycycline were added into the collagen gel as shown in Table 1. All the gels were cross-linked with 0.5% glutaraldehyde (reported to collagen dry substance) and hydrogels were obtained.

2.3. Rheological analysis of collagen hydrogels

The rheological measurements were performed with a rotational viscometer Multi-Visc Rheometer Fungilab, equipped with a standard spindle TR 9 and ultrathermostat ThermoHaake P5 maintained at 37^{o}C. The operating parameters for rheological experiments were previously described [28]. The viscosity of the hydrogels as a function of shear rate was recorded and the Power law model was applied for the evaluation of the flow properties:

$$\eta = m \cdot \dot{\gamma}^{-n} \quad (1)$$

where, m and n are parameters correlated with the designed hydrogels composition and determined through the linearization of eq. (1) by double logarithmic method [29]. Each rheological determination was carried out in triplicate.

2.4. Preparation of spongious composites

The cross-linked gels were frozen at -40^{o}C for 12 hours and then freeze-dried according to the method previously described [30] using the Christ Model Delta 2-24 LSC freeze-dryer (Germany). 3D spongious composites were obtained and characterized as follows.

Table 1

Compositions and codes of collagen hydrogels and correspondent matrices
Compoziţia şi codificarea hidrogelurilor colagenice

Code of hydrogels	Coll, %	DH*, %	PAW1**, %	GA**, %
Coll	1	0	0	0.5
Coll-P1	1	0	25	0.5
Coll-P2	1	0	50	0.5
Coll-P3	1	0	75	0.5
Coll-DH	1	0.2	0	0.5
Coll-DH-P1	1	0.2	25	0.5
Coll-DH-P2	1	0.2	50	0.5
Coll-DH-P3	1	0.2	75	0.5

* DH is reported to collagen gel volume
** PAW1 and GA are reported to collagen dry substance

2.5. FTIR spectroscopy

Infrared spectroscopy (IR) measurements were performed on an iN10 MX mid infrared FT-IR microscope operated in transmission, reflection or Ge-ATR mode. The spectra were recorded over the wavenumber range of 400–4000cm^{-1}by co-adding 32 scans with a resolution of 4cm^{-1} on finely crushed samples [31] .

2.6. Water absorption

To establish the water absorption, the scaffolds were firstly immersed in pure water at 37°C as we previously described [32]. At anticipated intervals, the samples were withdrawn and weighed. Using the following equation, the water absorption was calculated:

$$\% \text{ water up-take} = \frac{Wt - Wd}{Wd} \ (g/g) \qquad (2)$$

where, Wt denotes the weight of the samples at immersion time t and Wd denotes the weight of the dry samples. All samples were studied in triplicate.

2.7. In vitro degradation by collagenase

In order to investigate the enzymatic degradation of collagen scaffolds, mass loss was monitored as function of exposure time to a collagenase solution as we previously described [32]. Pieces of collagen scaffolds were immersed in a collagenase solution and incubated at 37°C. At fixed intervals, the swollen pieces were removed from the collagenase solution and weighed. The degradation percent was calculated using the following equation:

$$\% \text{ weight loss} = \frac{Wi - Wt}{Wi} \times 100 \ (g/g) \qquad (3)$$

where, Wi is the initial weight and Wt is the weight of the samples after a time t. All samples were studied in triplicate.

2.8. In vitro doxycycline release

The release kinetic experiments were carried out using a transdermal sandwich device adapted to a dissolution equipment, under paddle stirring conditions (Essa Dissolver, Italy), as we presented in detail in our reported papers [33]. Briefly, each spongious composite with doxycycline was placed into the sandwich device and then immersed in the release vessel. The release medium was phosphate buffer (7.4 pH), maintained at 37^{o}C. At fixed time intervals during twelve hours of experiments, sample of 5mL were extracted and replaced with same volume of fresh, preheated acceptor phase. The amount of drug released was evaluated by ultraviolet-visible spectrophotometry (Perkin-Elmer UV-Vis spectrophotometer) at 347 nm, using the calibration curve previously determined ($A_{1\%}^{1cm} = 204$, R^2= 0.9979) [34]. The general Power law model was applied to the kinetic data for characterizing the drug release mechanism:

$$\frac{Mt}{M\infty} = k \cdot t^n \qquad (4)$$

where, Mt/M∞ is the fraction of drug released at time t, k is the kinetic constant and n is the release exponent related to drug transport mechanism [33]. The kinetic parameters as well as the rheological ones were assessed using Table Curve 2D v5.01 software.

3. Results and discussion

The flow properties for collagen hydrogels without doxycycline and with different concentrations of PAW1 were monitored using stationary shear-rheometry. The rheograms recorded at 37^{o}C and plotted as viscosity versus shear rate are presented in Figure 1.

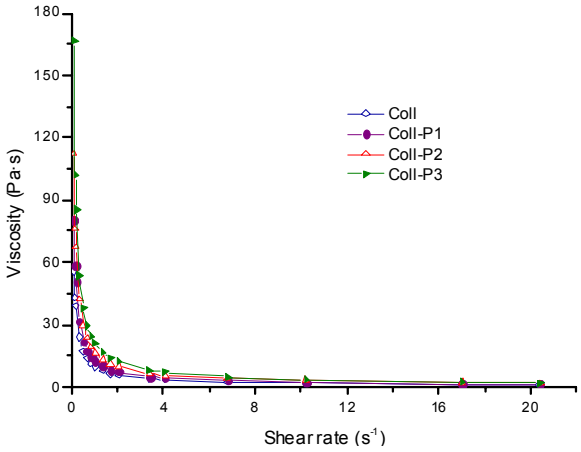


Fig. 1 - Plots of viscosity as a function of shear rate for the collagen hydrogels without doxycycline evaluated at 37°C / *Reprezentarea vâscozității în funcție de viteza de forfecare pentru hidrogelurile fără doxiciclină evaluate la 37°C.*

Analyzing the similar rheological patterns from Figure 1, it can be noticed that for all tested hydrogels the viscosity decreases with the shear stress increase. This type of curves corresponds to a non-newtonian pseudoplastic behaviour which facilitates the hydrogels flow [29], allowing their good manipulation and casting in trays during the preparation of spongious composites. These results are in accordance with our previous reported studies on the collagen hydrogels with different compositions [28,29].

The addition of different PAW1 proportions to collagen gels conducted to an increase of viscosity, the highest viscosity being obtained for the Coll-P3 sample with maximum amount of bioactive glass ceramic. The influence of PAW1 on collagen hydrogels without doxycycline is further quantified by fitting of the experimental data with Power law model (eq. 1). The Power law model

Table 2

The fitting parameters of the Power law rheological model for collagen hydrogels without doxycycline and with different PAW1 concentrations / *Parametri de fitare în modelul reologic al legii puterii pentru hidrogelurile cu colagen fără doxiciclină şi diferite concentraţii de PAW1*

Code of hydrogels	m	n	R^2
Coll	10.921	0.732	0.9914
Coll-P1	13.707	0.781	0.9961
Coll-P2	17.682	0.809	0.9990
Coll-P3	20.751	0.900	0.9992

fitting parameters for different concentration of PAW1 in collagen hydrogels are given in Table 2.

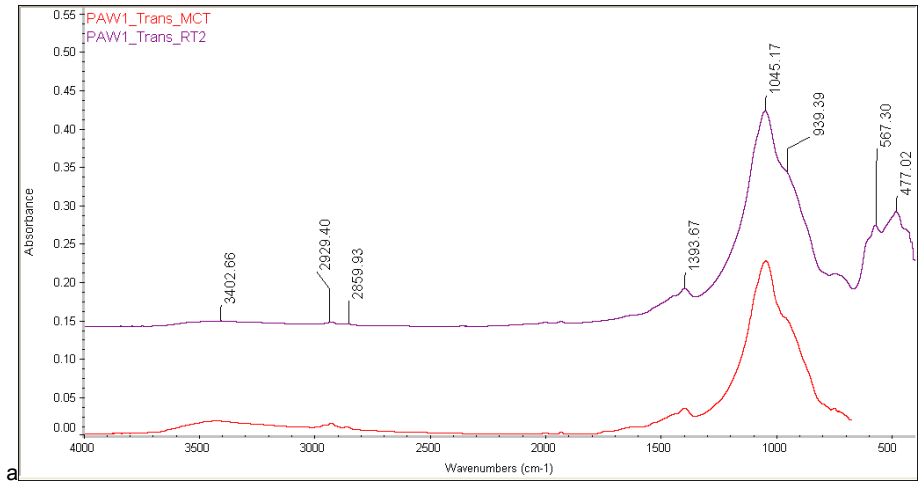
The values obtained for the determination coefficients R^2, ranging between 0.9914 and 0.9992 indicate that this rheological model fitted well the experimental data. The parameter m, associated with the viscosity obtained for the shear rate of $1 \cdot s^{-1}$ [29], presents the smallest value for the sample without PAW1. The addition of 25% PAW1 conducts to an m value increase of about 1.25 times compared to the sample without bioactive glass ceramic, while the doubling of the PAW1 amount in collagen hydrogel determines an m value increase of about 1.62 times. The maximum percentage of PAW1 leads to almost the doubling of m parameter value compared to the sample without bioceramics.

Both PAW1 and composite materials were characterized by FT-IR in order to identify the interaction between components.

In Figure 2 a-d the main absorption bands of collagen, PAW1 and their composites are presented.

The wollastonite (Figure 2a) showed bending modes of Si–O–Si and O–Si–O bonds at 477 cm^{-1}, the absorption bands centered at 1043 cm^{-1} and 939 cm^{-1}, attributed to the Si–O stretching vibration mode. The band at 3402 cm^{-1} is due to the absorption of moisture on the surface of the wollastonite [35].

The main absorption bands corresponding to collagen (Figure 2b) are: Amide A at 3308 cm^{-1}, Amide B at 2929 cm^{-1}, Amide I at at 1637 cm^{-1}, Amide II at 1547 cm^{-1} and Amide III at 1237 cm^{-1}.



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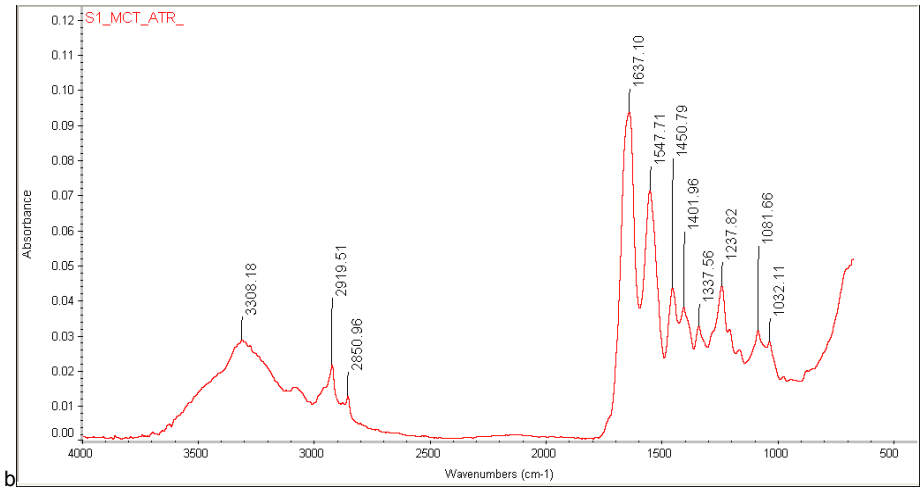

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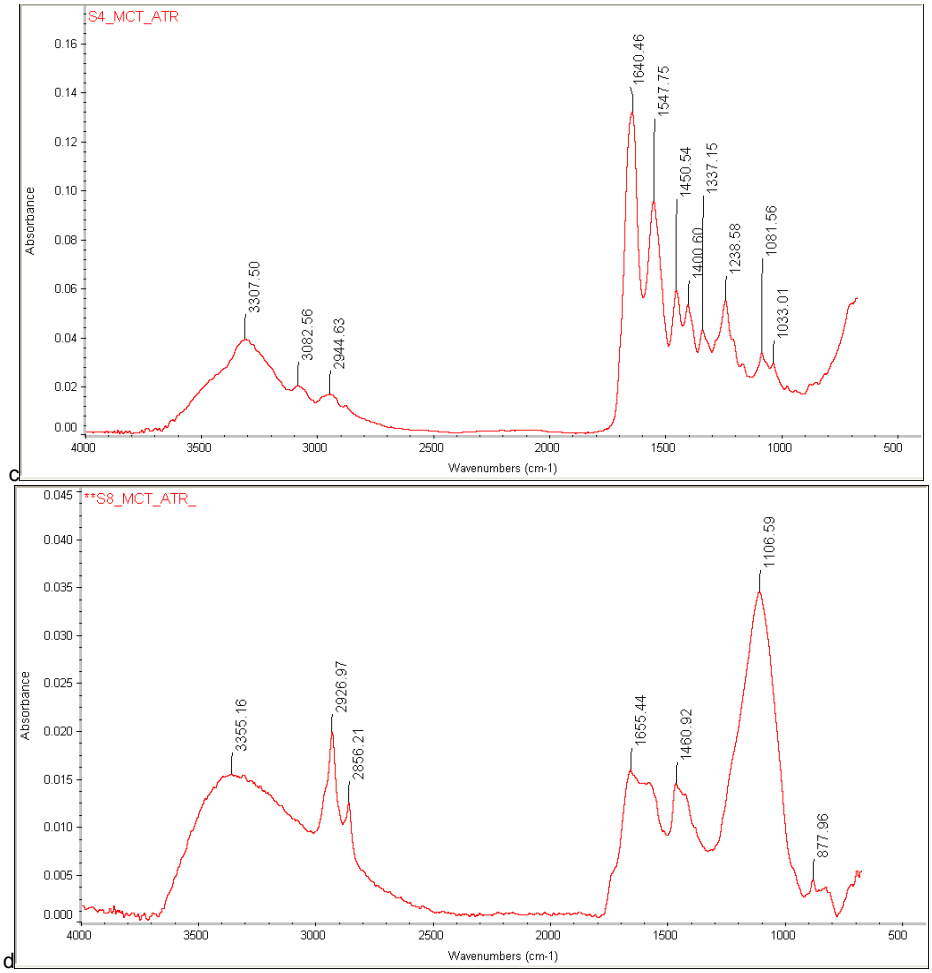

Fig. 2 - FT-IR spectra for/ *Spectrele FT-IR pentru:* a) PAW1; b) Coll; c) Coll-P3; d) Coll-DH-P3

As shown in the FTIR spectrum in Figure 2c, a distinctive peak at 1031 cm^{-1}, ascribed to the wagging vibration of covalent bond between carboxylic acid groups of collagen and calcium ions of wollastonite.

A visible influence of doxycycline on collagen-wollastonite composite could be seen in Figure 2d. Thus the amide II and III are disappearing and organic siloxane Si-O-C is formed, being assigned at 1106 cm^{-1}. Moreover, Amide I is shift from 1637 cm^{-1} to 1655 cm^{-1}.

The relative intensity of the main band of wolastonite, assigned to Si-O-Si vibration (~1100cm^{-1}) increases with the increase of the content of PAW1. The bands assigned to doxycycline cannot be identified due to its low content.

Collagen reference (Coll) which presented the highest pore size (Figure 3a) showed the highest amount of water absorbed (41.27%), meanwhile the denser structures especially the ones with the highest amount of wollastonite absorbed even 60% less than reference collagen. The wollastonite in the samples produces dense composites, and reduces water up-take ability. The results are in accordance with rheological behaviour. The addition of doxycycline increased the hydrophylicity, the water absorption being with 10% higher than for the corresponding reference sample. Although, because of interation between collagen, wollastonite and doxycycline, the water absorption is reduced in the sample with 50% wollastonite, the swelling being two times less than for the one without wollastonite.

Enzymatic degradation of collagen materials is dependent on, and determined by their triple helical integrity, by the degree of crosslinking and by the availability of cleavage sites [36]. *In vitro* degradation was performed in collagenase solution at 37^{o}C. As Figure 4a shows, after one hour collagen reference (cross-linked) were degraded about 15%. The degradation of composites with wollastonite and collagen are increasing with ceramic content increase, the most degradable being the one with 75% wollastonite (about 40% biodegradation). This is explained by the degradability properties of wollastonite and because the cross-linking agent is reported to collagen amount, not to composite volume. The samples which contain doxycycline are very stable in collagenase solution because doxycycline is collagenase inhibitor. The results are presented after 24 hours of degradation. The most stable is the one without wollastonite (Coll-DH – 44.06%) and the most degraded composite was the one

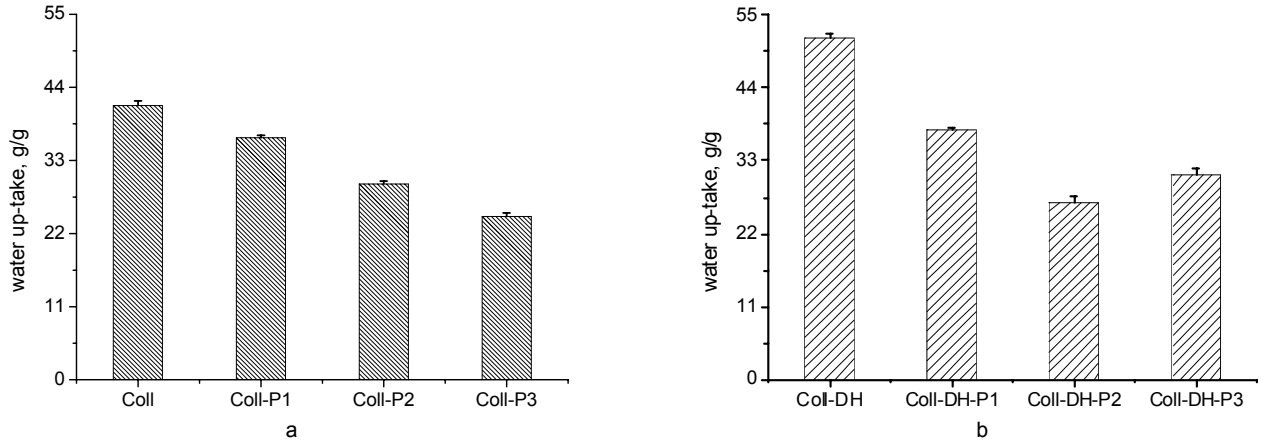

Fig 3 - Water up-take for collagen-wollastonite composites: a) without doxycycline; b) with doxycycline / *Absorbția de apă pentru compozitele pe bază de collagen-wolastonit: a) cu doxiciclină; b) fără doxiciclină.*

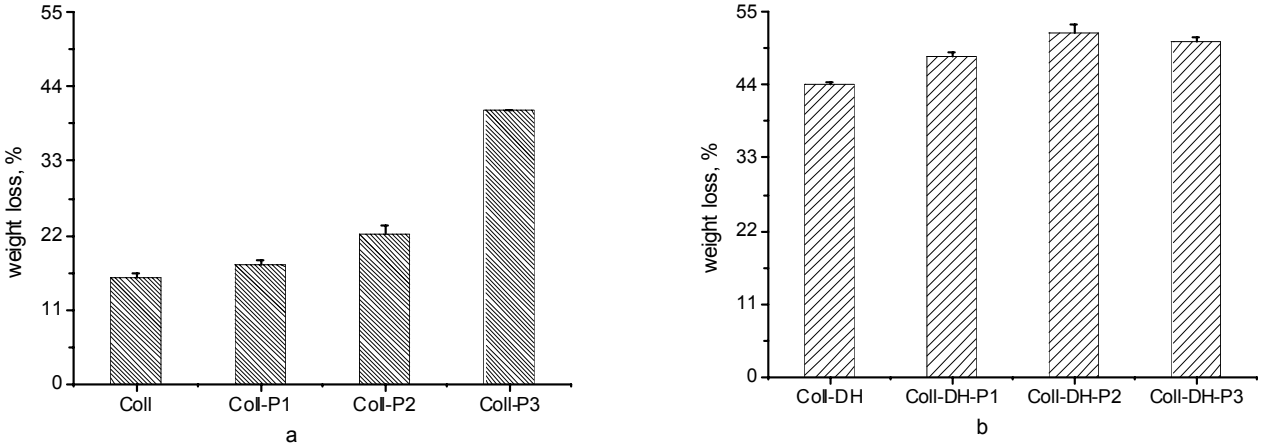

Fig 4 - *In vitro* degradation of composites: a) after 1 hour for composites without doxycycline; b) after 24 hours for composites with doxycycline / *Degradarea in vitro a compozitelor: a) după o oră pentru compozitele fără doxiciclină; b) după 24 de ore pentru compozitele cu doxiciclină.*

with 50% wollastonite (Coll-DH-P2).

Doxycycline delivery patterns from the spongious composites represented as released drug percent versus time are illustrated in Figure 5. The samples composition influences the drug release profiles. Figure 5 shows that the highest drug released percent was recorded for the spongious composites with 50% PAW1, while the smallest one was obtained for the sample without bioceramics. The release of doxycycline was smaller from the sample with 25% PAW1 compared to the one with 75% bioceramics.

An initial drug burst release was noticed in the first two hours of experiments, the percent released being between 19% and 43%. After this period, the drug is gradually released during the next ten hours. This kinetic behaviour is appropriate for prophylaxis and treatment of bone infected defects.

Table 3 indicates the fitting parameters specific to the Power law kinetic model.

From Table 3, it can be seen that all formulations fitted well this model, the values recorded for the determination coefficients R^2 being between 0.9879 and 0.9968. The values obtained for the release exponent indicate an anomalous non-Fickian mechanism based on a combination of drug diffusion and spongious composites erosion.

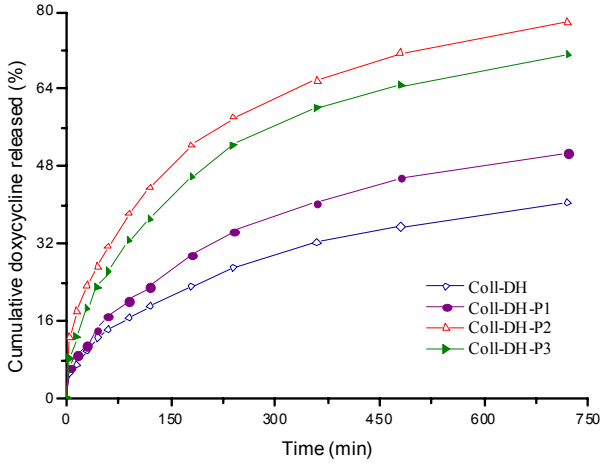

Fig. 5 - Cumulative doxycycline kinetic release profiles from spongious composites as function of time / *Profilele cinetice de cedare cumulate ale doxiciclinei din compozitele spongioase în funcție de timp.*

Table 3

The fitting parameters of the Power law kinetic model for spongious composites with different PAW1 concentrations
Parametri de fitare în modelul cinetic al legii puterii pentru compozitele spongioase cu diferite concentrații de PAW1

Code of spongious composites	Kinetic constant ($1/min^n$)	Release exponent	R^2
Coll-DH	0.024	0.432	0.9968
Coll-DH-P1	0.027	0.450	0.9930
Coll-DH-P2	0.072	0.369	0.9914
Coll-DH-P3	0.051	0.409	0.9879

4. Conclusions

Three-dimensional supports based on collagen and apatite – β-wollastonite without/with doxycycline hyclate were obtained under the form of hydrogels and spongious composites. The hydrogels with collagen and bioactive glass ceramics were rheologically assessed and a non-newtonian pseudoplastic behaviour was highlighted. The FT-IR spectroscopic determinations showed that the porous structure preserves the triple helicoidal structure integrity of native collagen. The composition of the scaffolds markedly influenced the water-uptake capacity, the resistance to the collagenase activity and the drug release kinetics. The percent of wolastonite leading to the maximum drug release was 50%. The results of this study indicated that the obtained composites are promising biomaterials for treatment and prevention of infected bone, further studies being recommended for testing their efficiency.

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