COLLAGEN-DOXYCYCLINE TOPICAL HYDROGELS: RHEOLOGICAL, KINETIC AND BIOCOMPATIBILITY STUDIES

MIHAELA VIOLETA GHICA[1], MĂDĂLINA GEORGIANA ALBU[2*], IRINA TITORENCU[3,4], LUMINIŢA ALBU[2], LĂCRĂMIOARA POPA[1]

[1] *"Carol Davila" University of Medicine and Pharmacy, Faculty of Pharmacy, Physical and Colloidal Chemistry Department, 6 Traian Vuia Str., 020956, Bucharest, Romania*
[2] *INCDTP – Division Leather and Footwear Research Institute, Collagen Department, 93 Ion Minulescu Str., 031215, Bucharest, Romania*
[3] *"Petru Poni" Institute of Macromolecular Chemistry, 41A Grigore Ghica Vodă Alley, 700487, Iasi, Romania*
[4] *"Nicolae Simionescu" Institute of Cellular Biology and Pathology, Cell Culture Department, 8 B.P. Hasdeu Str., 050568, Bucharest, Romania*
**corresponding author: albu_mada@yahoo.com*

Abstract

The aim of this study was the design of some topical drug delivery systems, as hydrogels, based on collagen and doxycycline hyclate, usable for the prevention and/or treatment of the infections appeared due to cutaneous wounds (superficial burns, surgical wounds). For this purpose the influence of the cross-linking agent (glutaraldehyde) on the rheological properties, release kinetics and the enzymatic biodegradability of the developed systems was followed. The biocompatibility with the human dermal fibroblast and endothelial cells for the most stable sample to the enzymatic degradation was also tested. Thus, all the designed hydrogels presented a non-newtonian pseudoplastic behaviour, the rheological analysis highlighting also that the viscosity increases in the same time with the cross-linking degree increase. The drug release from hydrogels follows the power law model. The cross-linking with glutaraldehyde reduced the doxycycline release from the drug system, but increased the resistance to the enzymatic degradation. The most stable hydrogel to the enzymatic degradation was the one cross-linked with 0.30% glutaraldehyde, and sustained human dermal fibroblast and endothelial cells growth.

Rezumat

Scopul acestui studiu a fost proiectarea unor sisteme de cedare topică a medicamentelor, de tip hidrogel, pe bază de colagen şi hiclat de doxiciclină, utilizabile în prevenirea şi/sau tratamentul infecţiilor apărute pe fondul unor leziuni cutanate (arsuri uşoare, răni chirurgicale). În acest scop s-a urmărit influenţa agentului de reticulare (glutaraldehida) asupra proprietăţilor reologice, cineticii de cedare şi biodegrabilităţii enzimatice a sistemelor proiectate. De asemenea s-a testat şi biocompatibilitatea cu fibroblaste şi celule endoteliale umane a celei mai stabile probe la degradare enzimatică. Astfel, toate hidrogelurile au prezentat o comportare pseudoplastică non-newtoniană, analiza reologică evidenţiind şi faptul că la creşterea gradului de reticulare creşte vâscozitatea acestora. Cedarea medicamentului din hidrogeluri respectă modelul legii puterii. Reticularea cu glutaraldehidă a redus eliberarea doxiciclinei din sistemul

medicamentos, dar a crescut rezistenţa la degradarea enzimatică. Cel mai stabil hidrogel la degradare enzimatică a fost cel reticulat cu 0,30% glutaraldehidă, acesta susţinând şi creşterea fibroblastelor dermale şi a celulelor endoteliale.

Keywords: hydrogel, collagen, doxycycline, rheology, release, biological studies

Introduction

Hydrogels are hydrophilic polymers [3,16] that have the ability to absorb large amounts of water, maintaining their tridimensional structure at the same time [7,8].

So far, various devices from both natural and synthetic polymers have been used as drug delivery systems [13,21]. Among them, the collagen is an attractive and suitable support for drug delivery, offering the advantage of a natural biomaterial with haemostatic and wound healing properties [15,19]. Besides its function as release support, collagen acts as a biological dressing for tissue regeneration in wound healing.

The vascularization and recovery of injured tissue represent currently a major problem because most biomaterials do not allow the development of blood capillaries. Important roles in wound healing have the endothelial and dermal fibroblasts, the way they respond to biomaterials being very important for the last ones further utilization. Together with endothelial cells (major cell for newly formed tissue vascularization in the healing process), dermal fibroblasts secrete extracellular matrix macromolecules, soluble factors that diffuse to the overlying epidermis and cytokines which have a role in neovascularization. As it is known, neovascularization is an important step in wound healing, and represents at the same time a defining process for the success of tissue regeneration [1,20].

For *in vivo* applications, the collagenic biomaterials suffer an enzimatic degradation, that's why they are stabilized through *in vitro* cross-linking [10]. The studies demonstrated that collagen cross-linked with a proper percent of glutaraldehyde is not toxic for different *in vitro* culture cells [9,17]. Collagen can be fully digested only by collagenases. Such enzymes are unique since they are able to cleave collagen triple helical region under physiological conditions of pH and temperature. The control of the degradation rate of collagen gels is an important aspect, as the *in vivo* resorption influences tissue regeneration ability [12].

Few drug delivery systems based on collagen were actually clinically tested and put on the medical market, although they have been intensely researched all over the world. Furthermore, these developments

bring about a better understanding of the benefits obtained from local delivery, as some new collagen-drug systems can replace the standard antibiotic systemic treatment [11]. The drug delivery systems based both on antibiotics and collagen can prevent topical infection and accelerate wound healing [4]. Doxycycline, a broad spectrum antibiotic, can be used to treat cutaneous bacterial infections and also inhibits the action of collagenase [14].

Thus, the aim of this study was to determine the drug release kinetics from different cross-linked and uncross-linked collagen supports. Furthermore the kinetic analysis was supplemented with the rheological study because it can offer useful informations from the biopharmaceutical point of view concerning the behaviour of the analyzed hydrogels at the application site. The *in vitro* biological tests were than performed by the collagenase-degradation and by the biocompatibility with fibroblasts and endothelial cells.

Materials and Methods

Chemicals

Type I collagen gel having a concentration of 2.67 % was obtained from calf hide by acid and alkaline treatments as previously described [18]. Collagenase type I, *C. histolyticum* was purchased from US Biological (USA), glutaraldehyde (GA) from Merck (Germany) and doxycycline hyclate (D) from Fluka, BioChemica, China. Chemicals used for cell cultures were obtained from Sigma (Germany) and those used for electron microscopy were supplied by Polysciences (USA), except sodium sulphate and lead citrate, which were obtained from Merck (Germany), and tannic acid from Mallinckrodt. Tissue culture flasks were purchased from Nunc (Germany). The human endothelial cell line, EA hy 926 (human aortic endothelial cells) and human fibroblasts cell line were obtained from the American Type Cell Culture Collection (ATCC). Sodium hydroxide and phosphate buffer solution (PB) pH 7.4 were of analytical grade.

Hydrogel preparation

The initial gel was adjusted at 1.2% collagen (C) and 7.2 pH with 1M sodium hydroxide under mechanical stirring. 0.2% (w/v) doxycycline hyclate was added to each gel. Collagen-doxycycline gels having 7.2 pH were cross-linked with 0, 0.15, 0.20, 0.25 and 0.30% glutaraldehyde, *reported to the dry collagen*, and then were stored for 24 h at 4°C for maturation or cross-linking processes. Depending on GA compositions, the obtained hydrogels were coded as follows: CD – the uncross-linked gel,

CD-0.15, CD-0.20, CD-0.25 and CD-0.30 – the gels cross-linked with the above mentioned concentrations of GA.

Rheological analyses

A rotational viscometer Multi Visc–Rheometer Fungilab with TR 9 standard spindle was used to determine the gel flow ability. The rheological experiments were performed at 23°C and 37°C (storage and kinetic experiments temperature), using a ThermoHaake P5 Ultrathermostat. The operational conditions were described in our previous studies [6].

In vitro release of doxycycline hyclate

The *in vitro* drug release kinetics from collagen hydrogels were evaluated using a “sandwich” device fitted to a USP dissolution apparatus in conjunction with paddle stirrers. The paddle was rotated with 50 rpm. The PB was used as release medium and maintained at 37°C. At certain time periods aliquots (5mL) were withdrawn from the release medium and replaced by the same volume of fresh pre-heated phosphate buffer solution. The amount of doxycycline hyclate released was spectrophotometrically evaluated at 347 nm using the calibration curve previously described [2].

The rheological and kinetic parameters were determined using TableCurve 2D v5.01 software.

In vitro degradation by collagenase

Enzymatic degradation of collagen hydrogels was investigated by monitoring the mass loss as function of exposure time by collagenase solution. 2g of collagen hydrogels were accurately weighed, placed in PB solution and collagenase (1μg/mL) and incubated at 37°C. At regular intervals the swollen scaffolds were removed from degradation solution, blotted dry and weighed.

Collagen-doxycycline hydrogels colonization

For *in vitro* colonization, the human endothelial cell line, EA hy 926 (human aortic endothelial cells) and human dermal fibroblasts cell line were grown in DMEM (Dulbecco’s Modified Eagle Medium) culture medium containing 4.5‰ glucose supplemented with 10% fetal bovine serum, and antibiotics (100U/l penicillin, 100U/l streptomycin, 50U/l neomycin). The hydrogels were sterilized for 24 hours with 70% ethanol, conditioned in culture medium for 24 hours and inoculated with endothelial cells and dermal fibroblasts (50.000 cells/mL). The cells on hydrogels were maintained in culture at 37°C in incubators with 5% CO_2 in air and high relative humidity (> 95%). All experiments were performed after 1 week of culture.

Hoechst staining

After one week in culture, the cells on collagen hydrogels were

washed in PB, fixed in 2% paraformaldehyde (one hour) and then cryoprotected. Specimens were frozen in liquid nitrogen and sectioned with a Leica CM 1800 cryotome; the thickness of the sections were 4-6 μm. The cryosections were washed with PB for 15 minutes, stained with Hoechst 33258 for 15 minutes, washed in distilled water, mounted in glycerol and examined with a Nikon microscope equipped with epi-fluorescence; the micrographs were captured with a Sony DSC-S75 Digital Camera [17].

Results and Discussion

For each hydrogel the rheogram shear stress (Pa) as a function of shear rate (s^{-1}) was built at 23°C and 37°C (Figure 1, A and B).

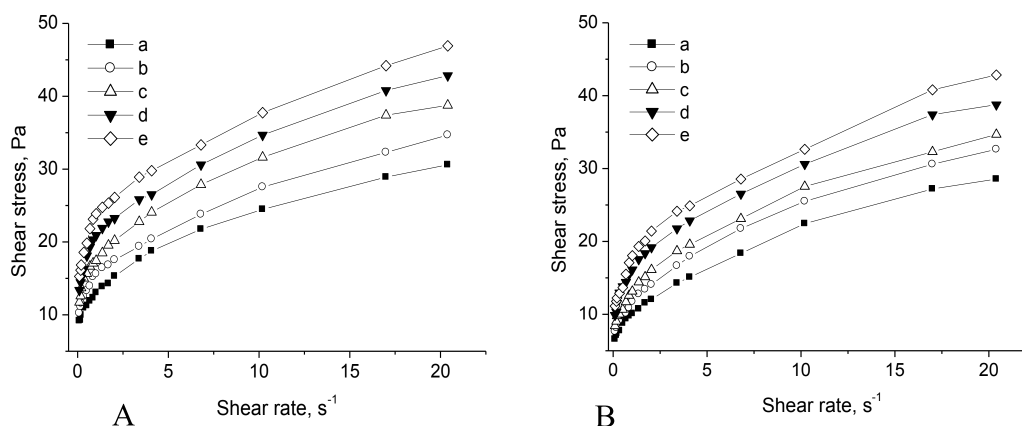

Figure 1
Rheograms for the designed hydrogels cross-linked with: a) 0%; b) 0.15%; c) 0.20%; d) 0.25%; e) 0.30% GA at A) 23°C and B) 37°C

By analyzing the rheological profiles in Figure 1, A and B, we can remark that all hydrogels present a non-nenewtonian behaviour at both temperatures. Different flow models were tested [2], the best value for the determination coefficient R^2 being obtained for Herschel–Bulkley model (equation 1), both at 23 °C and 37°C:

$$\tau = \tau_0 + K \cdot \dot{\gamma} \quad (1)$$

where, τ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s^{-1}), τ_0 is the yield stress (Pa), K is the consistency index ($Pa \cdot s^n$) and n is the flow index.

In table I the values of the rheological parameters specific to the above model for all hydrogel at both temperatures, as well as the determination coefficient values are given.

Table I
Values of rheological parameters given by the Herschel-Bulkley flow model at both temperature for collagen-doxycycline hydrogels

Collagen hydrogel	Yield stress Pa		Consistency index $Pa \cdot s^n$		Flow index		Determination coefficient	
	23°C	37°C	23°C	37°C	23°C	37°C	23°C	37°C
CD	6.651	5.238	6.361	4.796	0.442	0.531	0.9989	0.9983
CD-0.15	8.423	5.491	6.582	6.173	0.455	0.495	0.9975	0.9989
CD-0.20	9.175	5.682	8.294	7.432	0.425	0.452	0.9981	0.9988
CD-0.25	9.435	6.611	10.671	9.251	0.375	0.415	0.9959	0.9980
CD-0.30	10.134	7.082	12.495	10.114	0.351	0.416	0.9940	0.9959

The results presented in Table I showed that the increasing values of rheological parameters were recorded when GA concentration increases, due to the different physical or chemical bindings that can occur in the system.

Comparing the results given in the above table, it can be also observed that the values for the rheological parameters, for the all designed hydrogels, are higher for 23°C than 37°C, due to the destructuration induced by the temperature.

The values of the flow index n being inferior to 1 for all hydrogels at both temperatures, a pseudoplastic with shear thinning behaviour is highlighted, which facilitated the formulations flow and the formation of a continous film at the application site [6].

The rheological properties of the semisolid network described for each hydrogel give a certain kinetic release profile of the incorporated drug.

The kinetic profiles of *in vitro* doxycycline cumulative released from the uncross-linked and cross-linked gels are shown in Figure 2.

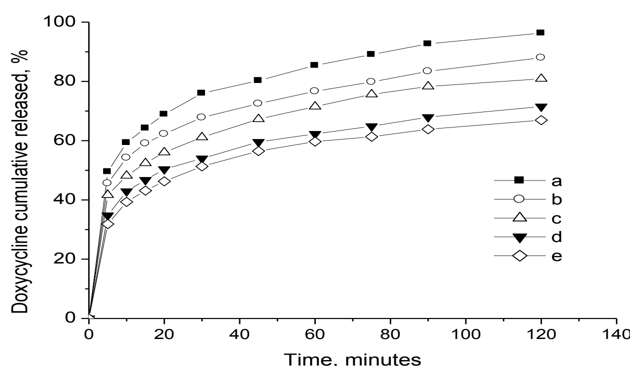

Figure 2
In vitro doxycycline release from the hydrogels cross-linked with: a) 0%; b) 0.15%; c) 0.20%; d) 0.25%; e) 0.30% GA

The release of doxycycline from gels depends on the cross-linking degree: the higher the amount of GA, the lower the percentage of doxycycline hyclate released, as shown in Figure 2. Thus, the cross-linked gel containing the maximum amount of GA releases 66.92% from the amount of doxycycline within 120 minutes, while the corresponding uncross-linked one releases 96.30% during the same period of time.

In order to establish the drug release mechanism different kinetic models were verified [2], the highest R^2 values being obtained for the power law model (Equation 2).

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

where, m_t / m_∞ is the drug fraction released at time *t*, *k* is the release rate constant incorporating structural and geometric characteristics of the delivery system, and *n* is the release exponent, indicating the release mechanism.

Estimated parameters from curve fitting to this model, as well as the determination coefficient values, are shown in table II.

Table II

Values of kinetic parameters given by the power law model for collagen-doxycycline hydrogels

Collagen hydrogel	**Release exponent (n)**	**Kinetic constant (k, %/min^n)**	**Determination coefficient**
CD	0.202	0.372	0.9946
CD-0.15	0.198	0.342	0.9977
CD-0.20	0.214	0.295	0.9970
CD-0.25	0.212	0.260	0.9946
CD-0.30	0.222	0.236	0.9912

The quantification of the cross-linking agent effect both on the doxycycline released from the selected hydrogel and on their flow properties was realized by setting out some quantitative relations kinetics-rheology (equations 3 and 4) [5]. For this purpose a non-linear estimation method with “user-specified regression” subroutine of Statistica^{TM}Stat software was used.

$$k = 4.14 \cdot \tau_0^{-1.47} \qquad (R = 0.9671) \qquad (3)$$

$$k = 0.96 \cdot K^{-0.59} \qquad (R = 0.9857) \qquad (4)$$

Pearson's coefficient (R) values for both equations indicate a good correlation between the release rate constant, *k*, and flow parameters, τ_0, K.

The resistance to the enzymatic degradation was studied for all the designed hydrogels.

The percent of hydrogel degradation was determined by the following relation (Equation 5):

$$\text{weight loss} = \frac{\left(W_i - W_t\right)}{W_i} \cdot 100 \qquad (5)$$

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

The experimental data were represented as residual mass digested (weight loss) during 4 hours and 24 hours respectively, according to the cross-linking agent concentration (Figure 3).

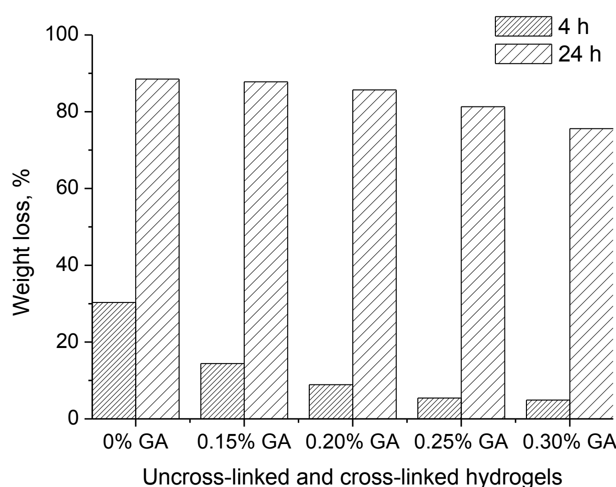

Figure 3
In vitro enzymatic degradation of collagen hydrogels

As shown in Figure 3, the uncross-linked hydrogel was digested 30.3% in 4 h and 88.5% in 24 h. The cross-linked samples were digested slower than the one uncross-linked. The hydrogels cross-linked with the higher content of GA (0.30%) was digested only 4.9% in the first 4 hours, while the one cross-linked with 0.15% GA was digested 14.3%. After 24 h all hydrogels showed very close value of weight loss (%) between 87.8 and 75.6%. Therefore, cross-linking with GA reduces the enzymatic degradation, which makes the amount of degraded collagen be lower in the same period of time.

Because the aim of our study was to develop drug delivery systems used in recovery of infected tissues, we then tested, from the biocompatibility with fibroblasts and human endothelial cells point of view,

the most stable hydrogel to the collagenase-degradation, namely the hydrogel cross-linked with 0.30% GA.

We observed that this hydrogel maintained its integrity in the culture medium. Than we followed the proliferation of the fibroblast and endothelial cells grown on the tested hydrogel, by phase contrast microscopy and the Hoechst staining (Figure 4).

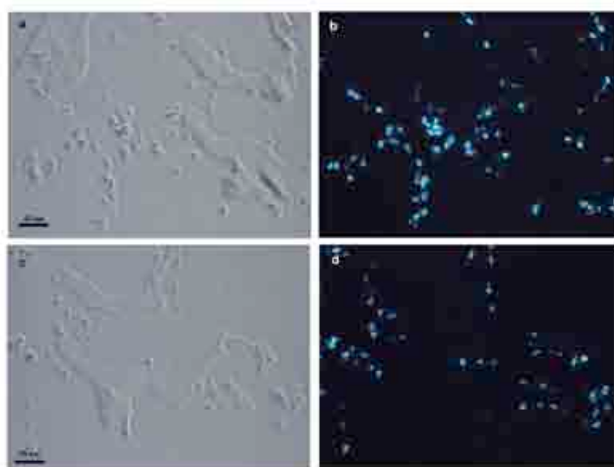

Figure 4
Human endothelial cells and fibroblasts cultured into collagen-doxycycline hydrogels: left panels: phase contrast microscopy, right panels: Hoechst staining; a, b – EA hy 926 grown on collagen-doxycycline hydrogels; c, d - human fibroblasts third passage grown on collagen-doxycycline hydrogels

Using the above technique, we have found that the collagen hydrogel sustained cellular growth. A homogeneous cell distribution was observed on this hydrogel and no significant differences were observed between the endothelial cells and fibroblasts grown on this hydrogel.

Conclusions

The particular characteristics of collagen hydrogels make them a adequate system for drug delivery. The cross-linking with glutaraldehyde decreases the amount of the released drug, but increases the collagen enzymatic degradation resistance. For the designed hydrogels the 0.30% of glutaraldehyde seems to be optimal for the above purposed. By our further studies of simultaneous modulation of the cross-linking agent and collagen concentration, the doxycycline hyclate release characteristics can be

modified in order to develop enzymatic degradation resistant and biocompatible delivery systems, with suitable flow and kinetic characteristics.

The equations kinetics-rheology set are important from the practical point of view because they can be extended for other glutaraldehyde concentration in order to determine the corresponding release rate constant and can be also used for other drugs with similar size and solubility.

Acknowledgements

One of the authors (IT) acknowledges the financial support of European Social Fund – "Cristofor I. Simionescu" Postdoctoral Fellowship Programme (ID POSDRU/89/1.5/S/55216), Sectoral Operational Programme Human Resources Development 2007 – 2013.

References

1. Ahlfors J.E.W., Billiar K.L., Biomechanical and biochemical characteristics of a human fibroblast-produced and remodeled matrix, *Biomaterials*, 2007, 28(13), 2183–2191.
2. Albu M.G., Ghica M.V., Popa L., Leca M., Trandafir V., *In vitro* kinetics release of doxycycline hyclate from collagen hydrogels, *Rev Roum Chimie*, 2009, 54(5), 373-379.
3. Anumolu S.S., Menjoge A.R., Deshmukh M., Gerecke D., Stein S., Laskin J., Sinko P.J., Doxycycline hydrogels with reversible disulfide crosslinks for dermal wound healing of mustard injuries, *Biomaterials*, 2011, 32(4), 1204-1217.
4. Elsner J.J., Zilberman M., Novel antibiotic-eluting wound dressings: An *in vitro* study and engineering aspects in the dressing's design, *J Tissue Viability*, 2010, 19(2), 54-66.
5. Ghica M.V., Physico-chemical and biopharmaceutical elements of semisolid systems with topical action. Applications to indomethacin hydrogels, 2010, *Printech, Bucharest*, 2010, 73-79.
6. Ghica M.V., Popa L., Moisescu Şt., Evaluation of some rheological parameters for sodium carboxymethylcellulose topical hydrogels with indomethacin, *Farmacia*, 2007, 55(6),671-679.
7. Gupta P., Vermani K., Garg S., Hydrogels: from controlled release to pH-responsive drug delivery, *Drug Discov Today*, 2002, 7(10), 569–579.
8. Hennink W.E., van Nostrum C.F., Novel crosslinking methods to design hydrogels, *Adv Drug Deliver Rev*, 2002, 54(1),13–36.
9. Ho H.O., Lin C.W., Sheu M.T., Diffusion characteristics of collagen film, *J Control Release*, 2001, 77(1-2), 97-105.
10. Khor E., Methods for the treatment of collagenous tissues for bioprostheses, *Biomaterials*, 1997, 18(2), 95-105.
11. Lee C.H., Singla A., Lee Y., Biomedical applications of collagen, *Int J Pharm*, 2001, 221(1-2), 1-22.
12. Mayne R., Burgeson R., Structure and function of collagen types, *Academic Press New York*, 1987.
13. Mihele D., Moldovan L., Mihele D., Dărmănescu D., Georgescu S.R., Determination on cell cultures of the proliferative action of some semisolid preparations for topical use with *Amygdali* oleum, *Farmacia*, 2010, 58(1), 102-107
14. Nicolescu A.C., Mircioiu C., Inhibition of a calcium-dependent cysteine protease by doxycycline, *Farmacia*, 2010, 58(2), 136-144

15. Ruszczak Z., Friess W., Collagen as a carrier for on-site delivery of antibacterial drugs, *Adv Drug Deliver Rev*, 2003, 55(12), 1679-1698.
16. Teles H., Vermonden T., Eggink G., Hennink W.E., de Wolf F.A., Hydrogels of collagen-inspired telechelic triblock copolymers for the sustained release of proteins, *J Control Release*, 2010, 147(2), 298–303.
17. Titorencu I., Albu M.G., Giurginca M., Jinga V., Antoniac I., Trandafir V., Cotruţ C., Miculescu F., Simionescu M., *In vitro* biocompatibility of human endothelial cells with collagen-doxycycline matrices, *Mol Cryst Liq Cryst*, 2010, 523, 82/[654] – 96/[668].
18. Trandafir V., Popescu G., Albu M.G., Iovu H., Georgescu M., Bioproduse pe bază de colagen, *Ars Docendi, Bucharest,* 2007, 102-103.
19. Wallace D.G., Rosenblatt J., Collagen gel systems for sustained delivery and tissue engineering, *Adv Drug Deliver Rev*, 2003, 55(12), 1631-1649.
20. Wong T., McGrath J.A., Navsaria H., The role of fibroblasts in tissue engineering and regeneration, *Brit J Dermatol*, 2007, 156(6), 1149–1155.
21. Zilberman M., Elsner J.J., Antibiotic-eluting medical devices for various applications, *J Control Release*, 2008, 130(3), 202–215.

Manuscript received: February 5th 2011