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M. G. Albu [a], M. V. Ghica [b], M. Leca [c], L. Popa [b], C. Borlescu [d], E. Cremenescu [d], M. Giurginca [e] & V. Trandafir [a]

[a] Collagen Department, Leather and Footwear Research Institute, Bucharest, Romania

[b] Faculty of Pharmacy, University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania

[c] Faculty of Chemistry, University of Bucharest, Romania

[d] SC ELMI PRODFARM SRL, Bucharest, Romania

[e] Faculty of Applied Chemistry and Material Science, University Politehnica of Bucharest, Bucharest, Romania

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Doxycycline Delivery From Collagen Matrices Crosslinked With Tannic Acid

M. G. ALBU,[1] M. V. GHICA,[2] M. LECA,[3] L. POPA,[2] C. BORLESCU,[4] E. CREMENESCU,[4] M. GIURGINCA,[5] AND V. TRANDAFIR[1]

[1]Collagen Department, Leather and Footwear Research Institute, Bucharest, Romania
[2]Faculty of Pharmacy, University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania
[3]Faculty of Chemistry, University of Bucharest, Romania
[4]SC ELMI PRODFARM SRL, Bucharest, Romania
[5]Faculty of Applied Chemistry and Material Science, University Politehnica of Bucharest, Bucharest, Romania

*Collagen-doxycycline matrices crosslinked with a natural polyphenol – tannic acid for the treatment of infected wounds were obtained by the freeze-drying of the corresponding gels. FT-IR spectra show that the triple helical structure of collagen is preserved in all the matrices, doxycycline produce collagen crosslinking and the degree of crosslinking increases with tannic acid concentration. Digestion of matrices using collagenase confirms the crosslinking effect of doxycycline and tannic acid and the increasing of the crosslinking degree with the amount of acid. The release of doxycycline from the matrices crosslinked with tannic acid is slower than that from the uncrossliked one and decreases with increasing of acid concentration, according to FT-IR and digestion results, and follows the power law model, with a release exponent of about 0.4, which indicates an anomalous transport. The matrices containing doxycycline, tannic acid and their combination do not develop gram-positive (*Staphylococcus aureus*) or gram-negative (*Escherichia coli, Pseudomonas aeruginosa*) bacteria, fungi or leavens.*

Keywords Antimicrobial effect; collagen matrices; doxycycline; drug delivery systems; tannic acid

Introduction

Collagen, the major constituent of connective tissues and the major structural protein of each organ is of particular interest as a natural polymer for obtaining drug delivery systems. It acts as a haemostatic and promotes the new tissue granulation and wound epithelization, functioning as a dressing for different types of wounds

Address correspondence to M. G. Albu, Leather and Footwear Research Institute, Bucharest, Romania. Tel.: (+4021)327.71.41; Fax: (+4021)323.52.80; E-mail: albu_mada@yahoo.com

[1–3]. However, collagen itself can not produce the healing of an infected wound because it is a protein in nature and bacteria can use it as a substrate [4]. But combined with suitable antibiotics highly efficient drug delivery systems for wound treatment can be obtained, due to a potential synergistic effect.

An ideal drug delivery system must show a low allergization quota, stability at body temperature, tissue compatibility, bactericidal activity, high bacterial resistance, broad activity spectrum, and low resorption rate [5].

Antibiotics local delivery has been studied successfully in clinics for aminoglycosides like gentamicin and tobramycin as well as for minocycline, tetracycline, teicoplanin or sulbactam-cefoperazone [6]. Although doxycycline is a bactericide for a broad spectrum of bacteria and inhibits the action of collagenase, so far it is found in very few drug delivery systems [7].

In order to improve the biochemical and mechanical properties of support and control the release of drugs, the collagen has to be crosslinked, the usual crosslinking agent being glutaraldehyde.

The aim of this study is the developing of collagen spongious matrices containing doxycycline as dressings for preventing and/or stopping the microorganisms' action on infected tissue or for prophylactic purposes. Besides the antibacterial, antienzymatic and haemostatic properties, the tannic acid – a natural polyphenol – can play the role of a crosslinking agent for collagen. The crosslinking effect of doxycycline and tannic acid on collagen was investigated by FT-IR and enzymatic degradation. *In vitro* doxycycline release from collagen matrices is also presented. The bactericidal effect of matrices was tested for both gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli, Pseudomonas aeruginosa*) bacteria, fungi or leavens.

Materials and Methods

The type I fibrillar collagen gel having a concentration of 1.2% (w/w) was extracted from calf hide by the technology currently used in the Research-Development Textile Leather National Institute Division Leather and Footwear Research Institute – Collagen Department [8]. Doxycycline hyclate was purchased from Sigma-Aldrich, China and tannic acid from Sigma-Aldrich, Germany. Sodium hydroxide and phosphate buffer solution, PBS (pH = 7.4), were of analytical grade. Type I collagenase obtained from *Clostridium histolyticum* was purchased from Sigma-Aldrich, Germany. The standard microorganisms *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, fungi and leavens were purchased from Sigma, Germany.

An amount of 0.2% doxycycline hyclate (reported to the collagen gel) was embedded under mechanical stirring into collagen gel having an initial pH of 2.5. The interaction between proteins and tannins being maximum in the vicinity of izoelectric pH, the pH of collagen gel was adjusted at pH 3.8 using 1 M sodium hydroxide. Then it was crosslinked with different amounts of tannic acid (0, 4, 5 and 10% reported to the dry collagen).

The collagen gels were freeze-dried using the Delta 2-24 LSC Christ, Germany lyophilizer.

The resulted matrices were named as shown in Table 1, depending on the presence of doxycycline or tannic acid and its amount.

IR spectra of the matrices were obtained using a FT-IR 6000 spectrophotometer equipped with ATR reflection system MKII Golden Gate Single (Jasco) in IR (MID and NIR) region with 150 acquisitions.

Table 1. Compositions of collagen matrices

Collagen matrix code	Collagen, %	DH, %	GA, %
M-B	1.2	0	0
CD-B	1.2	0.20	0
CD-0,15	1.2	0.20	0.15
CD-0,20	1.2	0.20	0.20
CD-0,25	1.2	0.20	0.25
CD-0,30	1.2	0.20	0.30

The enzymatic degradation was carried out in physiological conditions (PBS having pH 7.4, 37°C) using collagenase. About 1 g of matrix was incubated in 0.5 mL collagenase solution for 36 h. The reaction was stopped by cooling the samples at 0°C. What resulted was centrifuged for 15 min, the supernatant freeze-dried and reported to the initial weight of samples (g/g).

In vitro release of doxycycline hyclate was determined in triplicate at 37 ± 0.5°C using a modified USP paddle method (''sandwich'' device). The paddle was rotated with 50 rpm. The phosphate buffer having the pH 7.4 was used as release/dissolution medium (200 mL). Aliquots of 5 mL were withdrawn from the reaction medium at different times and replaced with the same volume of fresh pre-heated phosphate buffer. The amount of doxycycline hyclate released was determined spectrophotometrically at 347 nm using the calibration curve [7].

The microbial contamination was assessed according to the standards COLIPA – Guidelines on Microbial Quality Management [9]. Collagen matrices with an area of $2\,cm^2$ were evaluated in duplicate by inoculation on specific culture medium, followed by subcultures and identification of pathogenic microorganisms by the determination of the total number of aerobic microorganisms or fungi or by biochemical tests for identification of pathogenic microorganisms. The results were expressed as presence (UFC/cm^2) and absence of bacteria.

Results and Discussions

The FT-IR spectra of the control collagen matrix, shown in Figure 1, presents bands specific for the type I non-denatured collagen: amide A due to N-H stretching vibration (A_A) at $3299\,cm^{-1}$ and amide B at $2936\,cm^{-1}$, amide I at $1634\,cm^{-1}$ (strong) assigned to the stretching vibrations of the C=O groups of amide groups in proteins [10], amide II at $1548\,cm^{-1}$(strong) arising from the N-H bending vibration strongly coupled with the C-N stretching one of the protein amide groups, amide III centered at $1238\,cm^{-1}$ assigned to the bending vibration of N-H with significant mixing with the CH_2 wagging vibration from the glycine backbone and proline side chains [10].

FT-IR is able also to emphasize the interaction between collagen and doxycycline and/or the crosslinking with tannic acid. Thus, the modifications of collagen structure can be estimated by the following semi-quantitative correlations:

–ratio A_I/A_A, correlated with the collagen crosslinking: the higher A_I/A_A ratio the more advanced the crosslinking;
–ratio A_I/A_{II} correlates with the degree of hydrolysis;

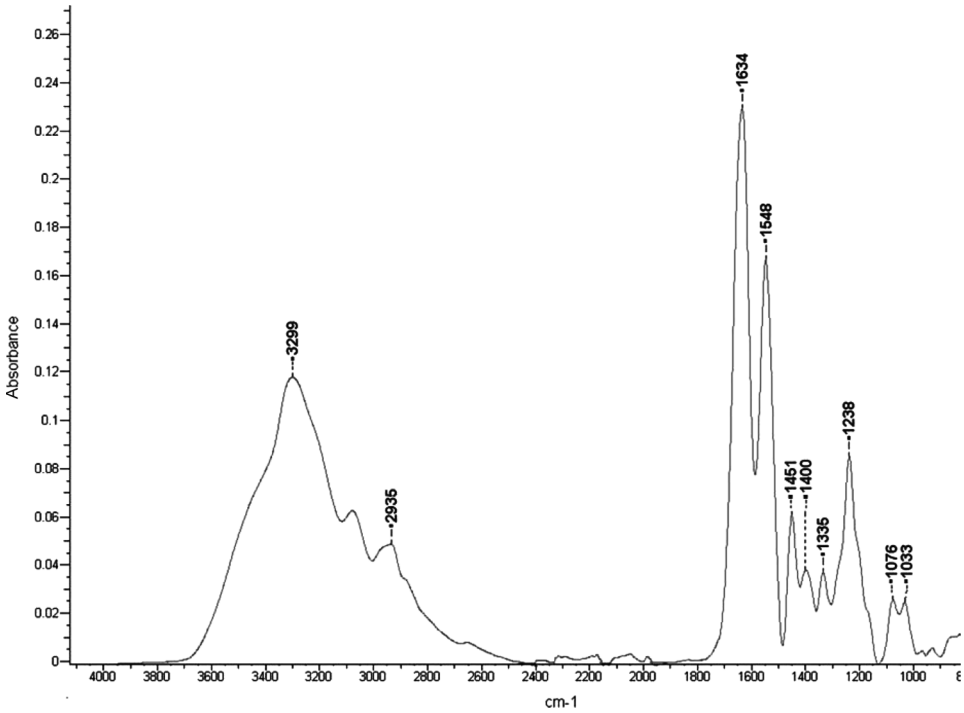

Figure 1. FT-IR spectra of collagen matrix M-A.

–ratio A_{III}/A_{1450} is a measure of preservation of the integrity of the triple helical structure of collagen, values lower than unity indicating denaturing [11];
–difference between amide I and II frecquences, $\Delta\nu = (\nu_1 - \nu_2)$, gives information about the presence of denatured collagen [12], values higher than 100 indicating denaturing.

The effects of doxycycline or doxycycline and tannic acid on the above mentioned rates and difference are shown in Table 2.

The value of the ratio A_I/A_A increases when doxycycline is added as Table 2 shows, which proves that it has the capacity to crosslink collagen, while tannic acid produces the reduction of this ratio, which can be interpreted as an interaction between doxycycline and tannic acid. But increasing the amount of tannic acid

Table 2. Characteristic ratios and difference of the collagen matrices from Table 1

Collagen matrix	A_I/A_A	A_I/A_{II}	A_{III}/A_{1450}	$\Delta\nu$ $(\nu_1 - \nu_2)$
M-A	1.96	1.38	1.40	86
CD-A	2.00	1.62	1.26	84
CD-T4	1.88	1.59	1.31	86
CD-T5	1.92	1.55	1.35	87
CD-T10	1.96	1.55	1.30	86

produces the increase of this ratio, that is of the degree of crosslinking. The higher crosslinking was obtained as expected, for 10% tannic acid.

All the values of the ratio A_{III}/A_{1450} are higher than unity, which demonstrates that the triple helical structure of collagen is preserved in each matrix. This is confirmed also by the $\Delta\nu$ values, which are smaller than $100\,cm^{-1}$, ranging between 84 and $87\,cm^{-1}$. Thus, it can be concluded that neither doxycycline nor tannic acid modifies the triple helical structure of collagen at pH 3.8.

If the IR bands of the matrices M-A and CD-A are compared, it can be seen that the band from $3299\,cm^{-1}$ shifts with $7\,cm^{-1}$, which means that hydrogen bonds form. Also, $\Delta\nu_{M\text{-}A}$ is higher than $\Delta\nu_{CD\text{-}A}$ that may indicate that the associations of CO-NH groups is more pronounced for CD-A.

The effect of tannic acid on ν_{OH} is shown in Figure 2, in which the ν_{OH} shift as a function of tannic acid concentrations is represented. The frequency of bands decreases linearly with the amount of tannic acid, which supports the conclusion that the degree of crosslinking increases with increasing amount of tannic acid and that the collagen matrix containing 10% acid posses the highest degree of crosslinking.

The degradation rate of a collagen matrix is a very important aspect, *in vivo* resorption time influencing the ability of regeneration of a tissue. In *in vitro* experiments collagenase is the most used enzymes for collagen digestion [13]. It cleaving only the collagen chains and not the intermolecular bonds, biodegradation with collagenase allows also the evaluation of the degree of crosslinking of the collagen from matrices.

The resistance of the studied collagen matrices at enzymatic degradation with collagenase in physiological conditions (PBS with pH 7.4, 37°C) is shown in Figure 3.

It was obtained that the reference collagen matrix is the most digested −95% within 36 hours (it is completely digested in 42 hours). The presence of doxycycline reduces the digestion to 32% – about three times lower than that of M-A. The amount of digested collagen decreases when tannic acid is added, the rate of biodegradation

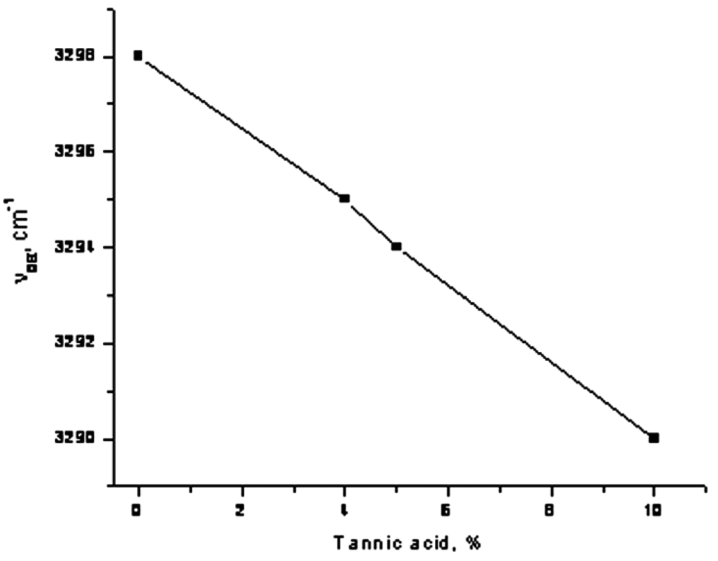

Figure 2. Shifts of ν_{OH} as a function of tannic acid concentrations.

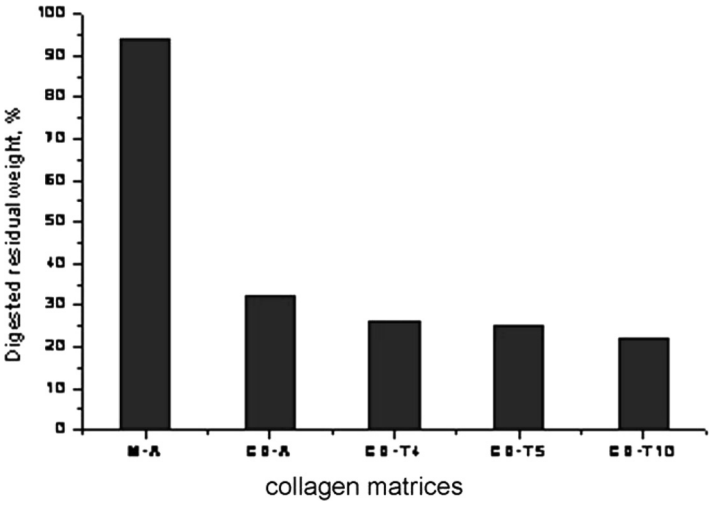

Figure 3. Resistance of collagen matrices at digestion 36 h with collagenase.

being slower for the crosslinked collagen matrices and decreasing slowly with tannic acid concentration. Thus, the most resistant to digestion is the matrix containing doxycycline a 10% tannic acid. This can be explained both by the crosslinking effect of the tannic acid and doxycycline and their capacity to inhibit collagenase.

The drug release was performed both for the uncrosslinked and crosslinked collagen matrices containing doxycycline in order to evaluate the amount released to the infected tissue and the release mechanism. The release profiles of doxycycline from the collagen matrices from Table 1 are presented in Figure 4.

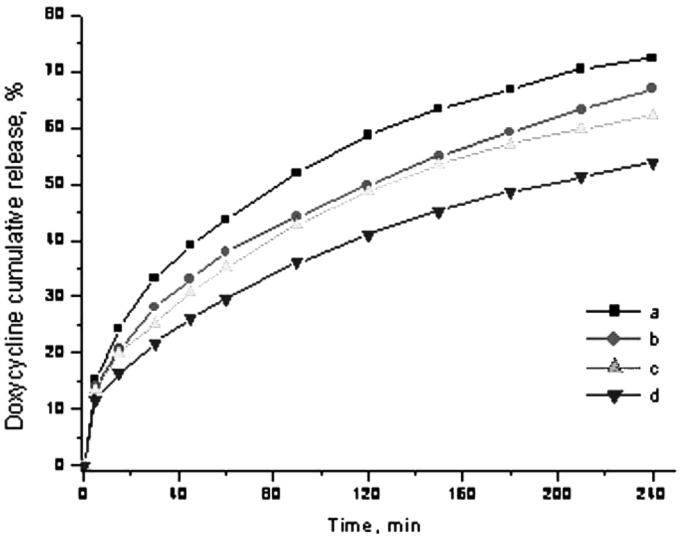

Figure 4. *In vitro* release of doxycycline from collagen matrices at 37°C: a – CD-A; b – CD-T4; c – CD-T5; d – CD-T10.

The Figure 4 shows that tannic acid produces the decreasing of the amount of doxycycline released and that the increasing of its percentage magnifies this effect, as expected considering the increasing of crosslinking with the amount of tannic acid. Thus, the matrix crosslinked with 10% tannic acid allows the releasing of the lowest amount of doxycycline −47.6% in 240 min, whereas the percentage of releasing for the uncrosslinked matrix (CD-A) is 72.4% during the same time. The matrices containing 4 and 5% tannic acid release 62.1 and 66.9% doxycycline respectively, their degrees of crosslinking being very close.

The results for the doxycycline release are in close agreement with the spectral characteristics and enzymatic degradation data and demonstrate that both doxycycline and tannic acid crosslink the collagen.

To study the doxycycline release kinetics, the data obtained from *in vitro* experiments from Figure 4 were fitted with the Higuchi, zero order and power law kinetics models [7]. The values of the correlation coefficients show that the mechanism is governed by power low equation:

$$\frac{m_t}{m_\infty} = k \cdot t^n \tag{1}$$

where m_t is the amount of drug released at time t, m_∞ – total drug content in the designed collagen matrix, m_t/m_∞ – fractional release of the drug, k – kinetic constant and n – release exponent, indicating the mechanism.

The values of the kinetics parameter for the collagen matrices containing doxycycline and doxycycline and tannic acid from Table 1 are presented in Table 3.

The values of the release exponent n were calculated as the slope of the straight lines fitting the release data using the least-squares methods. The n exponents have very close values, of about 0.4 while the kinetic constant decreases when tannic acid is introduced and has the smallest value for the matrix having the higher degree of crosslinking, as expected.

The FT-IR data, enzymatic biodegradation and doxycycline release show that the most convenient matrix is CD-T10 which contains 10% tannic acid.

Biomaterials being, by definition, intended to be used on or in areas of the body where the defenses against infection are most likely to be impaired, assurance of their microbial quality represents a crucial issue. That is why the antibacterial activity of the matrices M-A, CD-A and CD-T10 was determined using the most encountered bacteria – *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, fungi and leavens – *candida*, *cryptococus*, *histoplasma* şi *malassezia*. In order to have a

Table 3. Experimental values of kinetic parameters for doxycycline release from matrices

Collagen matrix	n	k, $\%/s^n$
CD-A	0.407	0.01540
CD-T4	0.411	0.01291
CD-T5	0.413	0.01202
CD-T10	0.411	0.01045

Table 4. Microbiological characteristics of collagen matrices

Collagen matrix	Characteristics				
	Total number of aerobic microorganisms (UCF/cm^2)			Total number of fungi and levurs (UCF/cm^2)	Results
	S. aureus	*E. coli*	*P. aeruginosa*		
M-A	1	1	Absent	Absent	2 UFC/cm^2 – Rejected
CD-A	Absent	Absent	Absent	Absent	Allowed
CD-T10	Absent	Absent	Absent	Absent	Allowed
CT-10A	Absent	Absent	Absent	Absent	Allowed

good evaluation of the bactericidal activity of the tannic acid in collagen matrices the microbiological characteristics of the matrix containing 10% tannic acid – CT-10A – were also determined. The results for all of them are given in Table 4.

As can be seen from the Table, the control collagen matrix developed both *S. aureus* and *E. coli*, while those containing doxycycline (CD-A), tannic acid (CT-10A) or their combination (CD-T10) did not develop any of the tested microorganisms. On one side this is due to the presence of doxycycline and on the other one to tannic acid, both being antimicrobial substances.

Conclusions

Collagen matrices containing doxycycline, uncrosslinked or crosslinked with tannic acid, were obtained from proper gels at pH 3.8 by the freeze-drying process. The FT-IR spectra show that the triple helical structure is preserved in all the studied matrices and the crosslinking increases with tannic acid concentration. The control collagen matrix is digested 95% by collagenase within 36 hours whereas the digestion of that containing doxycycline is only 32% and of those containing doxycycline and tannic acid ranges between 25 and 22% – depending on the degree of crosslinking – during the same period of time. The release of doxycycline from the matrices crosslinked with tannic acid is slower than that from the matrix containing only doxycycline, but in all the cases the power law model is followed, with a release exponent of 0.4 that indicates an anomalous transport process. Both doxycycline and tannic acid eliminate the development of microorganisms on collagen matrices.

Acknowledgments

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