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COLLAGEN-THUJA TINCTURE BIOMATERIALS FOR WOUND TREATMENT. 4. POROUS MATRICES CONTAINING THUJA TINCTURE AND CHLORHEXIDINE DIGLUCONATE

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Collagen-based biomaterials containing *Thuja occidentalis var.* columnaris tincture and chlorhexidine digluconate (CHDG) prepared as hydrogels having pHs 3.8 or 7.4 and containing all the combinations between 0.5, 1.0 and 1.5 mL tincture/100 g hydrogel and 0.02, 0.05 and 0.10 % CHDG were transformed into matrices by liophylization. Ratios A_{III}/A_{1450} and differences (νA_I - νA_{II}) shown by FT-IR bands prove that the used combinations do not disturb the triple helical conformation of collagen, but the cross-linking reduces gently with increasing of CHDG concentration. SEM images of matrices obtained from acid hydrogels emphasize very irregular pores with large distribution and pretty compact matrices, especially for low CHDG concentration, suggesting that the collagen is protected from cross-linking by components from tincture. The matrices resulted from slight basic hydrogels are more compact, suggesting a more advanced cross-linking, excepting the one containing the highest amount of CHDG, for which the hydrogels become again homogenous and the image looks close to that obtained from acid hydrogels. The amounts of CHDG released from the matrices obtained from acid and slight basic hydrogels are comparable, excepting the first 90 min during which the first ones deliver little higher amounts.

INTRODUCTION

Collagen porous matrices were designed as permanent wound dressings or scaffolds for tissue engineering, their porous structure canalizing the fibroblast migration from the wound edge, which results in the production of newly synthesized extracelular matrix by cells.[1]

The most used method to obtain convenient collagen matrices is the freeze-drying of collagen solutions or hydrogels.[2,3] So pretty expensive, it gives the most homogenous matrices, the size and form of pores being controlled by the ice crystals produced during the freezing process.

The size and form of ice crystals can be controlled by the collagen concentration, its pH, temperature and speed of freezing. Thus, a rapid freezing at (-80)^{o}C results in small pores of uniform size (about 15 μm), while freezing at temperature ranging between (-40) and (-20)^{o}C gives larger and less homogenous pores of 25-110 μm;[4] a low pH results in smaller pores, effect prominent especially if the freezing temperatures ranges between (-35) and (-20)^{o}C.[5] The optimal pore size for wound dressings ranges between 50 and 150 μm, providing the appropriate space for cellular infiltration and proliferation.[6]

Collagen being a significant component of the extracellular matrix, the porous matrices have very wide application: as haemostatic and wound covering materials,[7-9] scaffolds in tissue engineering,[9] delivery systems for cells proteins, drugs – including antibiotics[10-13] – and nucleic acids.[14-17]

Considering the beneficial effect of type I collagen in wound healing, the bacteriostatic (antiseptic) activity of CHDG and bactericide

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action of Thuja tincture, the matrices containing the named component can be very efficient for wound healing.

The purpose of the present paper is the study of the effects of freeze-drying, pH, CHXD and Thuja tincture concentrations on collagen native conformation from the porous matrices containing 0.02, 0.05 and 0.10% CHDG and 0.5, 1.0 and 1.5 mL Thuja tincture/100 g hydrogel for each CHDG concentration by FT-IR and of their morphology by SEM. The influence of pH, Thuja tincture and CHXD concentration on kinetics of CHXD delivery from the matrices using a modified USP paddle method is also presented.

RESULTS

The superposed FT-IR spectra of the collagen matrices obtained from the acid hydrogels matured for 24 h at 4°C are shown in Fig. 1.

The ratios of absorbencies of FT-IR bands A_{III}/A_{1450} and the differences (νA_I - νA_{II}), cm^{-1}, able to evaluate the integrity of collagen triple helical structure, as well as of the ratios A_I/A_A, used to appreciate the extent of collagen cross-linking are given in Table 1.

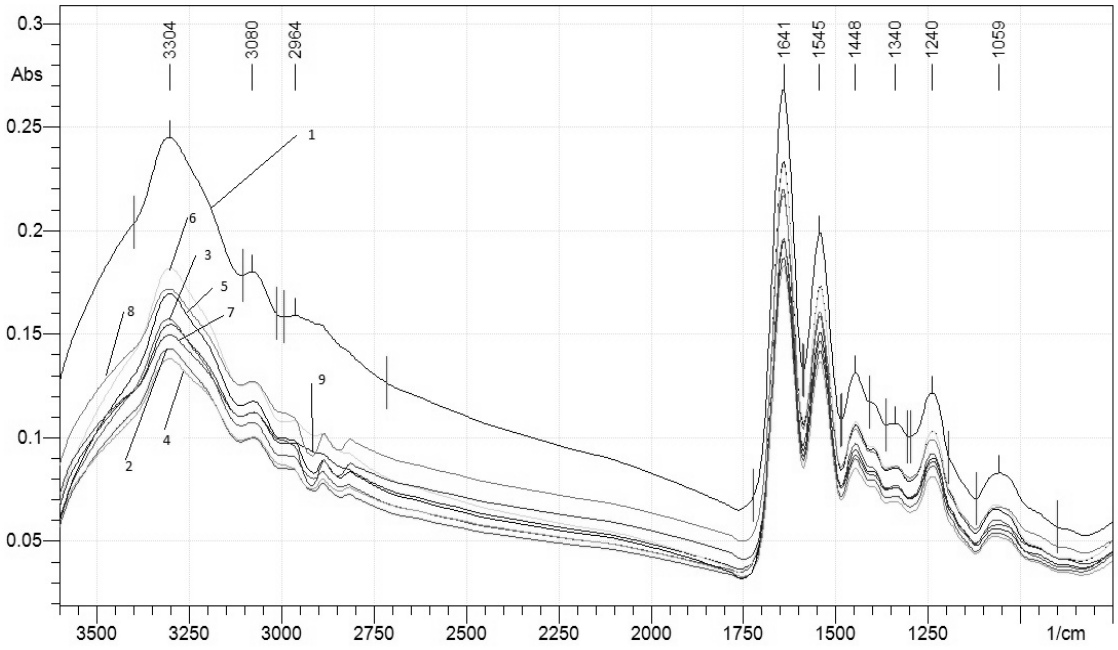


Fig. 1 – Superposed FT-IR spectra of collagen matrices obtained from collagen hydrogels having pH 3.8 containing 0.02% DGCH and: 1 – 0.5, 2 – 1.0 and 3 – 1.5% tincture; 0.05% DGCH and: 4 – 0.5, 5 – 1.5 and 6 – 1.5% tincture; 0.10% DGCH and: 7 – 0.5, 8 – 1.0 and 9 – 1.5% tincture.

Table 1

Ratios A_{III}/A_{1450}, A_I/A_A and differences (νA_I - νA_{II}), cm^{-1} and A_I/A_A obtained for the matrices prepared from acid collagen hydrogels containing the specified amounts of CHDG and Thuja tincture

Thuja tincture, mL/100 g hydrogel	A_{III}/A_{1450}	A_I/A_A	(νA_I - νA_{II}), cm^{-1}
	0.02% CHDG		
0.5	3.60	1.25	100
1.0	3.33	1.27	96
1.5	1.18	1.20	96
	0.05% CHDG		
0.5	2.66	1.43	100
1.0	2.34	1.26	100
1.5	2.16	1.20	100
	0.10% CHDG		
0.5	2.57	1.46	100
1.0	1.21	1.57	96
1.5	2.01	1.24	96

The FT-IR spectra of the collagen matrices obtained from the slight basic hydrogels matured for 4 h at 4°C are very similar with those of the acid ones shown in Fig. 1.

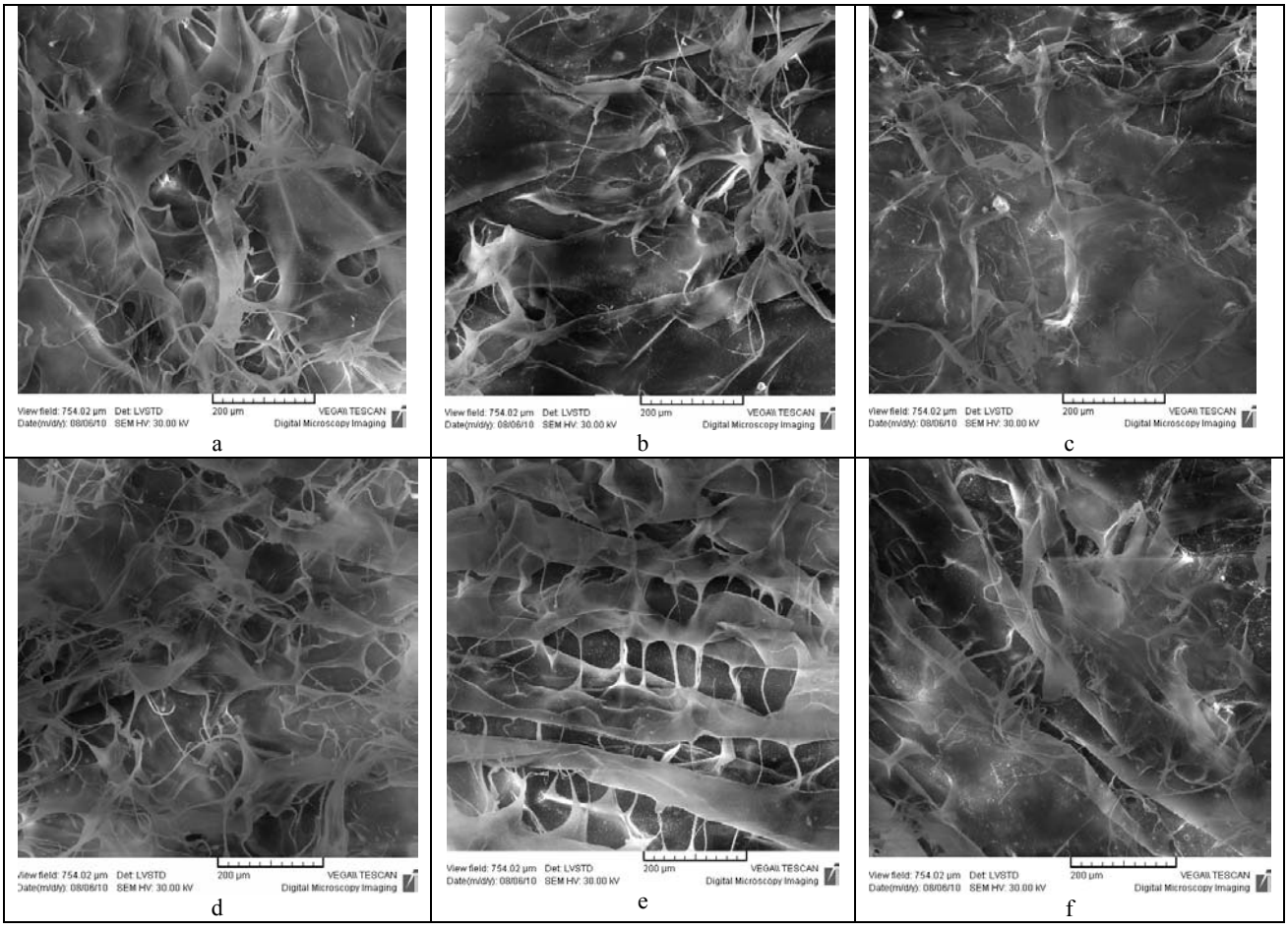
The values of the A_{III}/A_{1450} and A_I/A_A ratios and (νA_I - νA_{II}) differences are presented in Table 2.

SEM images of the matrices obtained from the acid hydrogels and having the amounts of Thuja tincture and CHDG given in Table 1 are presented in Fig. 2a-i.

Table 2

Ratios A_{III}/A_{1450}, A_I/A_A and differences (νA_I - νA_{II}), cm^{-1} and A_I/A_A obtained for the matrices prepared from slight basic collagen hydrogels containing the specified amounts of CHDG and Thuja tincture

Thuja tincture, mL/100 g hydrogel	A_{III}/A_{1450}	A_I/A_A	(νA_I - νA_{II}), cm^{-1}
	0.02% CHDG		
0.5	3.33	1.17	100
1.0	3.05	1.22	96
1.5	3.14	1.03	96
	0.05 % CHDG		
0.5	4.82	0.90	100
1.0	3.94	0.91	100
1.5	4.07	1.10	100
0.5	2.40	0.83	96
1.0	2.84	1.14	96
1.5	2.74	0.93	97



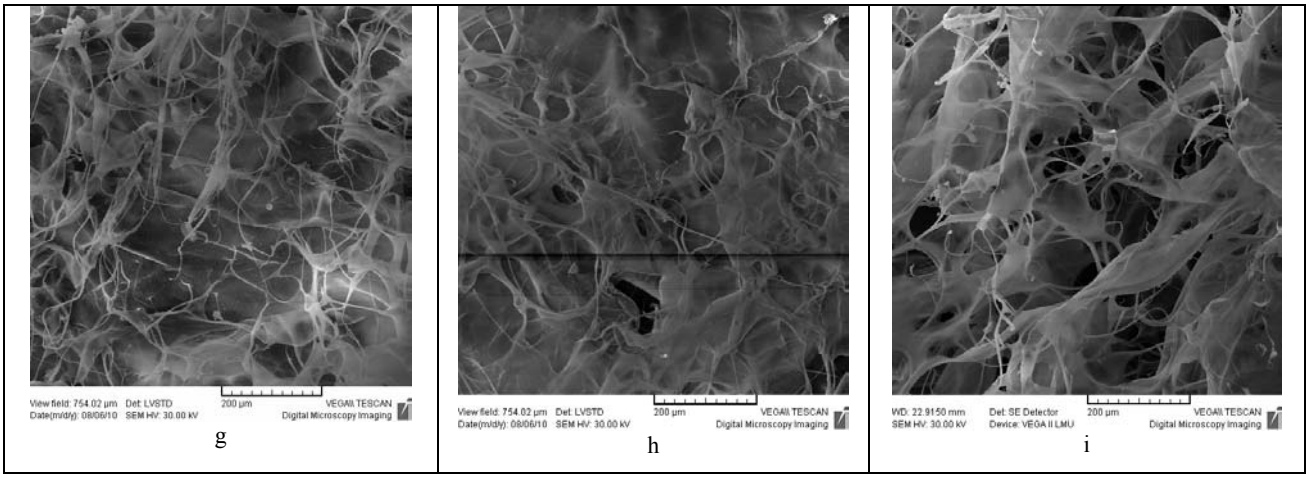
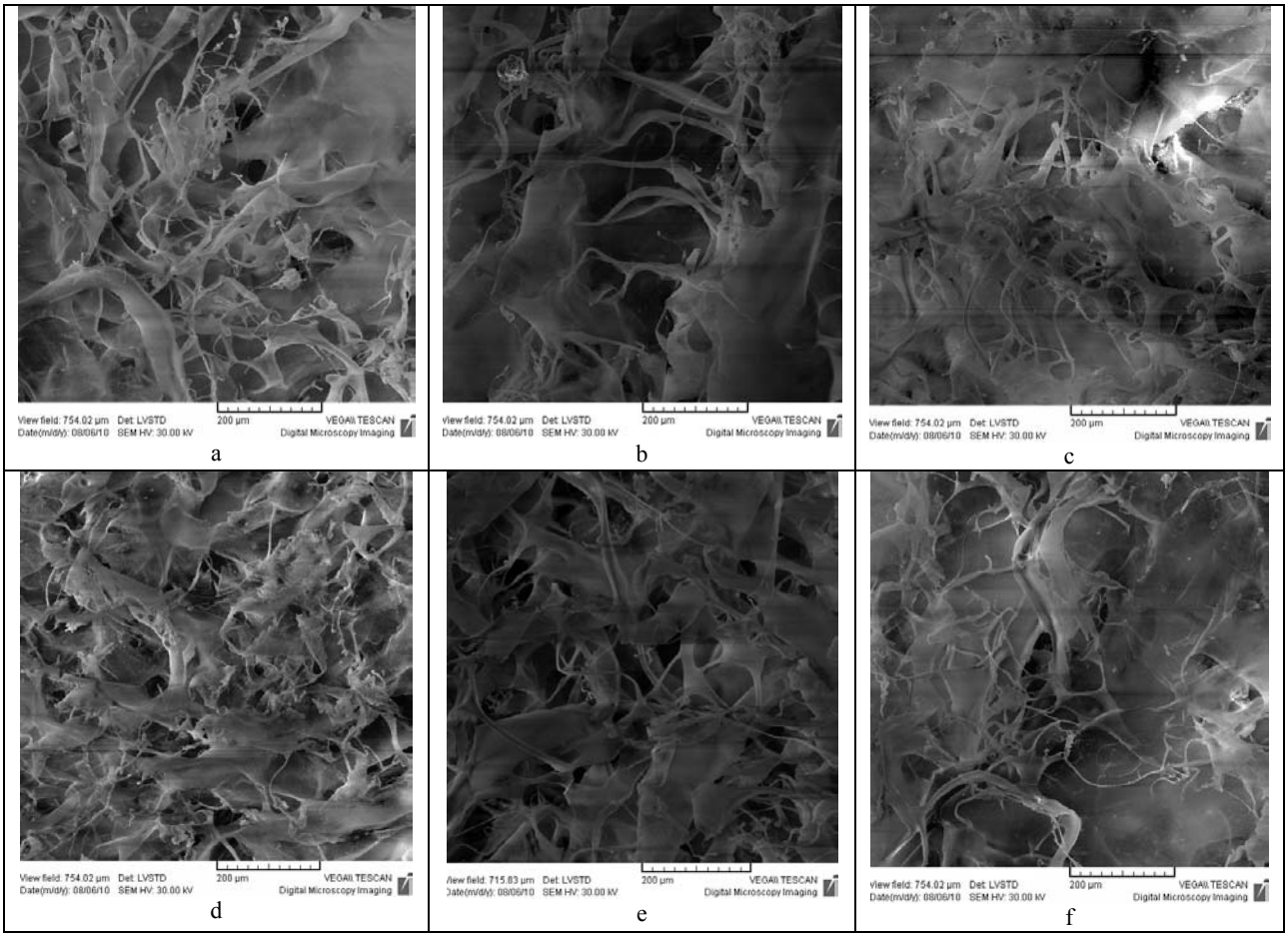


Fig. 2 – SEM images, 200x, of matrices obtained from the acid hydrogels containing: 0.02% DGCH and a – 0.5, b – 1.0 and c –1.5 mL tincture/100 g hydrogel; 0.05% DGCH and d – 0.5; e – 1.0 and f – 1.5 mL tincture/100 g hydrogel; 0.10% DGCH and g – 0.5; h – 1.0 and i – 1.5 mL tincture/100 g hydrogel.

In Fig. 3a-i SEM images of the matrices obtained from the slight basic hydrogels and containing the specified amounts of Thuja tincture and CHDG are given.

The curves representing the cumulative release of CHXD from the three series of collagen porous matrices obtained from the acid and slight basic hydrogels are shown in Fig. 4a, b respectively.



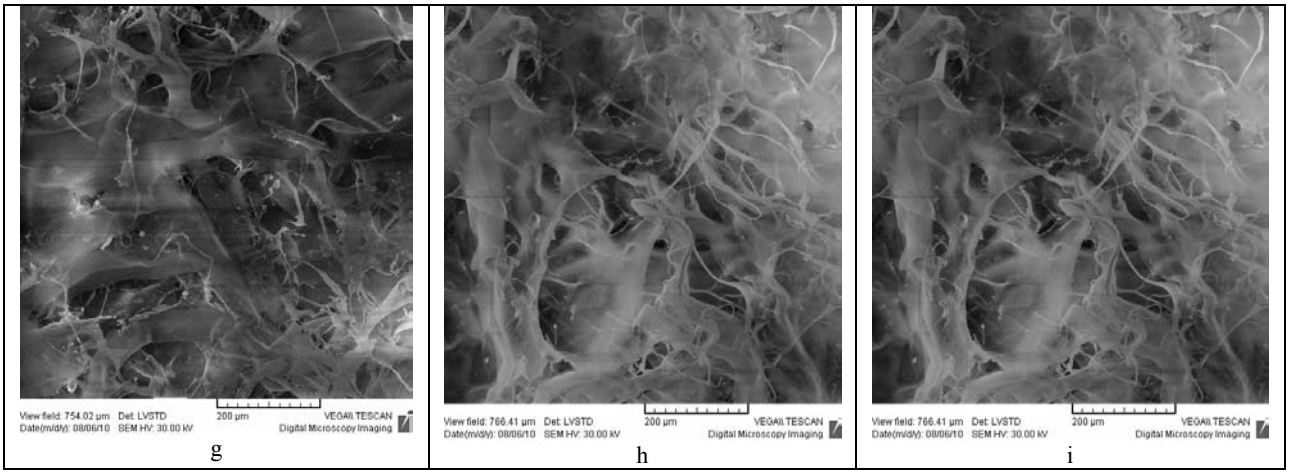


Fig. 3 – SEM images, 200x, of matrices obtained from the slight basic hydrogels containing: 0.02% DGCH and a – 0.5, b – 1.0 and c –1.5 mL tincture/100 g hydrogel; 0.05% DGCH and d – 0.5; e – 1.0 and f – 1.5 mL tincture/100 g hydrogel; 0.10% DGCH and g – 0.5; h – 1.0 and i – 1.5 mL tincture/100 g hydrogel.

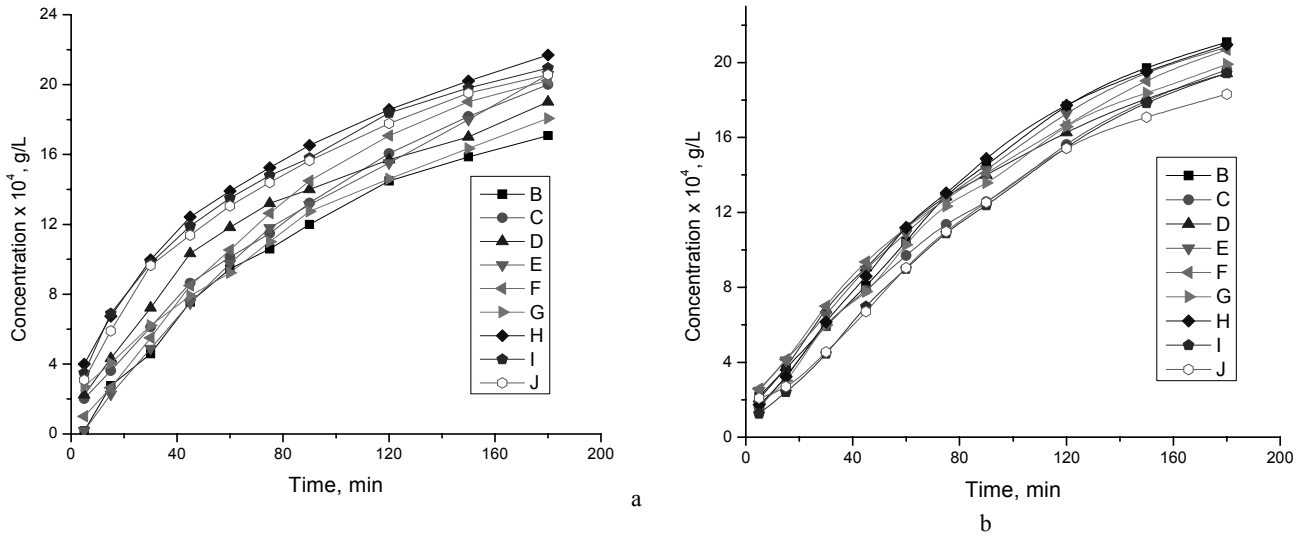


Fig. 4 – Cumulative release of CHDG from a – matrices obtained from acid hydrogels and b – matrices from slight basic ones containing: $2x10^{-2}$% CHDG and B – 0.5, C – 1.0, D – 1.5 mL tincture/100 g hydrogel; $5x10^{-2}$% DGCH and the same amounts of tincture (E, F, G respectively); 10^{-1}% DGCH and the above amounts of tincture (H, I, J).

DISCUSSION

The collagen hydrogels containing the amounts of Thuja tincture and CHDG given in Table 1 and 2 remain homogenous after maturation (24 h at 4°C) when the pH is 3.8, while those having pH 7.4 undergo, starting with about 4 h, the syneresis phenomenon[18] as the hydrogels containing only Thuja tincture do.[19] That is why the last hydrogels were introduced into the lyophilizer after 4 h of maturation. This can signify that the components from tincture interact more strongly with collagen than CHDG.

FT-IR spectra of the collagen matrices obtained from the acid hydrogels, presented superposed in Fig. 1, show that all the vibrational bands characteristic to peptide groups important for establishing of collagen conformation, presence of denatured collagen and extent of cross-linking[20] have the same frequencies in the limits of the experimental errors (4 cm^{-1} – the spectral interval between the data points).

The ratios A_{III}/A_{1450}, higher than unity, demonstrate that the nine mixtures between CHDG and Thuja tincture do not affect the triple helix conformation of collagen. So the values decrease with increasing of tincture amount as Table 1 shows, they remain higher than unity. The differences (νA_I - νA_{II}) range between 96 and 100 cm^{-1}, which justify the affirmation that the matrices do not contain denatured collagen.

The ratios A_I/A_A decrease slightly with increasing of tincture amount inside each series, that is the tincture reduces the cross-linking, but

the differences are small, while CHDG produces a weak increase.

All the bands obtained for matrices resulted from the slight basic hydrogels are weaker that those given by the acid ones, but the variation of bands frequencies are within the experimental errors.

The ratios A_{III}/A_{1450} are all higher than unity, as can be seen in Table 2, with the lowest values for the series containing the maximum amount of CHDG, whilst the differences (νA_I - νA_{II}) are lower than 100 cm^{-1} for the series containing 0.02 and 0.10% CHDG and higher for those with 0.05%.

The values of the ratios A_I/A_A are very close to those given by the matrices obtained from acid hydrogels for 0.02% CHDG and lower for 0.05 and 0.10%, which means that the cross-linking is comparable for the matrices containing the minimum amount of CHDG and lower when its amount increases, which indicates that in slight basic medium CHDG reduces a bit the cross-linking by tincture components.

SEM images of matrices obtained from acid hydrogels given in Fig. 3a-i show that the minimum concentration of CHDG and the three amounts of Thuja tincture result in completely different morphology compared with those containing only one of the components:[18,19] the pore are very irregular in shape and size that increase with tincture amount, probably due to the cross-linking produced by tincture components, which makes the matrices more compact. Increasing the CHDG concentration at 0.05% the pores become more definite and have closer size for the first two amounts of tincture and the matrix becomes compact only for the highest concentration. The more regular morphology of matrices containing larger amounts of CHDG suggests that it protects collagen from the cross-linking produced by components from tincture, reacting them probably in acid medium and blocking thus the cross-linking. The less compact SEM images of the matrices containing 0.1% CHDG from Fig. 2g-i confirm this hypothesis.

SEM images of matrices obtained from slight basic hydrogels matured for 4 h, presented in Figure 3a-i, are quite different from those obtained from the acid hydrogel: they are more compact at a given concentration of tincture and CHDG, which means that cross-linking is more intense at pH 7.4 than at 3.8, excepting the highest amount of CHDG, for which the hydrogels become again homogenous. The morphology of the last series of matrices looks closer to that obtained from the corresponding acid hydrogels, but the agglomerates are larger, showing that collagen cross-linking is more extended.

The cumulative delivery of CHDG from the matrices obtained from the acid hydrogels, presented in Fig. 4a, shows that the rates of CHDG release are significant over the whole time range on which the determinations were made, so they decrease slightly with time. The higher the initial CHDG concentration the higher the amount delivered for all the amounts of tincture used. Increasing of amount of Thuja tincture has as effect a very slight decrease of CHDG released especially for long times. Thus, the amounts of delivered antiseptic range between $1.6x10^{-5}$ g/L for the matrices containing the minimum amount of CHDG and tincture (curve B) and $4.0x10^{-4}$ g/L for that containing the maximum amount of CHDG and minimum quantity of tincture (curve H), while the maximum amounts vary between $1.31x10^{-3}$ and $2.17x10^{-3}$ g/L (the same curves, respectively). The releasing of CHDG form the matrices obtained from slight basic hydrogels is different as figure 4b shows: it increase almost linearly with time up to about 100 min, then the increasing reduces steadily, but the minimum amount delivered is higher and the maximum one a bit lower and depend less on CHDG and Thuja tincture concentration than in the case of the first matrices: it varies between $1.25\ x10^{-4}$ and $2.57x10^{-4}$ g/L for 5 min (curves I and F respectively) and between 1.82 and $2.11x10^{-3}$ g/L for 3 h (curves J and D respectively). Thus, the CHDG delivery from the matrices resulted from the hydrogels having pHs 3.8 and 7.4 are comparable, excepting the first 90 min, in which the first matrices release a little more amounts due to a slighter cross-linking. It must be remembered that the last matrices were obtained after 4 h of maturation, which resulted in a reduced crosslinking by components from tincture.

EXPERIMENTAL

Collagen hydrogels were prepared as described in a previous paper,[18] by diluting the initial 1.83% (w/w) collagen hydrogel with pH 2.39 with proper amounts of distilled water, 1M sodium hydroxide, 4% CHDG solution (FAGRON, Germany) and Thuja tincture (prepared in our laboratory from fresh ground leaves and twigs[21]) under stirring. The **matrices** were obtained by hydrogels' lyophilization in conditions given in paper.[19] The acid hydrogels were matured at 4°C for 24 h while the basic ones only for 4 h to avoid the syneresis phenomenon.

FT-IR and **SEM** measurements were made using the same equipment and conditions as in the papers.[19]

The measurement of ***in vitro*** **release of chlorhexidine digluconate** was described in a previous paper.[18]

CONCLUSIONS

FT-IR spectra show that the nine combinations between Thuja tincture and CHDG used do not disturb the triple helical conformation of collagen from the matrices obtained by the liophylization of the hydrogels having the pHs 3.8 and 7.4. So the ratios A_{III}/A_{1450} decrease a bit with increasing of tincture amount within each series, they remain higher than unity. The assumption is also supported by the absence of denatured collagen. The cross-linking reduces slightly with increasing of CHDG concentration.

SEM images of matrices obtained from acid hydrogels demonstrate that the morphologies of the matrices containing the mixtures of the two components are completely different from those of the matrices containing only one of them. The pores have very irregular shape and size and matrices become more compact, especially when CHDG concentration is low, suggesting that it protect the collagen from cross-linking by components from Thuja tincture. The matrices obtained from slight basic hydrogels are more compact at a given concentration of tincture and CHDG, proving a more intense cross-linking, excepting the highest amount of CHDG, for which the hydrogels become again homogenous and the matrices look more close to those obtained from acid ones.

The matrices with Thuja tincture and CHDG obtained from acid and slight basic hydrogels release comparable amounts of antiseptic, but those released from the first are a little higher within the first 90 min due to a more reduced crosslinking, so the slight basic hydrogels were matured only for 4 h before liophylization, compared with 24 h of maturing in the case of the acid ones. The higher the initial CHDG concentration the higher the amount delivered for all the amounts of tincture used for the first matrices, while increasing of amount of Thuja tincture has as effect a very slight decrease of CHDG released especially for long times. In the case of the second series of matrices the amount of CHDG released depend less on tincture and antiseptic concentrations.

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