CHARACTERIZATION AND IN VITRO RELEASE OF CHLORHEXIDINE DIGLUCONATE CONTAINED IN TYPE I COLLAGEN POROUS MATRICES

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Porous collagen matrices containing chlorhexidine digluconate (CHXD) were obtained by freeze-drying of three series of 1.1% collagen hydrogels having the pHs 3.8, 7.4 or 7.4 plus 0.15% glutaraldehyde (GA) reported to the collagen from hydrogel, each series containing 0, 0.02, 0.05 or 0.10% CHXD reported to the amount of gel. FT-IR spectra show that freeze drying, pH, CHXD or/and GA do not affect the triple helical conformation of collagen molecules forming fibrils. CHXD increases a bit the crosslinking of matrices obtained from acid hydrogels irrespective of its concentration, while of those resulted from slight basic ones is reduced to a slight extent in the absence of GA and much more in its presence, which means that the crosslinking decreases with CHXD concentration increase due to its reaction with the carboxyl groups of collagen and GA respectively. Matrices’ morphology reflects the changes produced in hydrogels by CHXD and/or GA: those obtained from acid hydrogels have lamellar structure, which becomes denser with increasing of CHXD concentration, orientation disappears in the matrices obtained from slight basic hydrogels, CHXD produces the agglomeration of fibrils that increases with CHXD concentration and GA enhances it. In the matrices containing 0.1% CHXD a part of it separates due to the regression of its solubility in basic media. The amounts of CHXD released from the matrices increase with CHXD concentration irrespective of pH and GA presence. The lowest amounts are released by the matrices obtained from the hydrogels with acid medium and the highest ones from the non-crosslinked matrices obtained from hydrogels with slight basic pH containing 0.1% CHXD.

INTRODUCTION

Type I collagen is the most investigated natural polymer as a drug delivery matrix due to its high biocompatibility, low antigenecity,1,2 controlled biodegradability by crosslinking,7 possibility to be processed in different forms such as gel, membrane, sponge and fiber and the easiness to which form composites with other natural and synthetic polymers or ceramics.3,5 In biological systems, collagen promotes cell attachment, proliferation, differentiation, organogenesis, tissue regeneration, and wound healing.5

Collagen hydrogels of proper concentration can be transformed into three-dimensional sponges, also called porous matrices or scaffolds. The most used process to manufacture relatively homogenous porous collagen devices proved to be solution or hydrogel lyophilisation.7,8 The size and structure of pores are determined by the size and form of ice crystals produced during the freezing process before lyophilisation. These can be monitored by collagen concentration and pH, temperature and speed of freezing. It was shown9 that rapid freezing at –80°C resulted in small pores of uniform size (about 15 µm), while freezing at temperatures ranging between –20 and –40°C gave less homogenous larger pores of 25-110 µm. A low pH resulted in smaller pore size, effect more obvious at freezing temperatures ranging between –35 and –20°C.10

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A collagen matrix serves as an analog of the extracellular matrix, acting as a physical support structure and as an insoluble regulator of biological activity that affects cell processes such as migration, contraction, and division. Collagen being a significant component of the extracellular matrix, such porous matrices have a wide number of applications: haemostatic and wound covering materials, scaffolds in tissue engineering, delivery systems for cells, proteins, drugs and nucleic acids on a predictable and long-term basis. They also promote cell and tissue attachment and growth.

Recent studies have shown the feasibility and, which is more important, the benefits of implants delivering antibiotics.

Chlorhexidine digluconate containing collagen hydrogels having the pHs 3.8 and 7.4, the last non-crosslinked or crosslinked with 0.15% glutaric aldehyde, characterized and used as CHXD delivery systems, discussed in a previous paper, were transformed by freeze-drying into porous matrices. The effects of freeze-drying, pH, CHXD concentration, GA as well as of GA and CHXD on collagen triple helical conformation from the three series of porous matrices were studied by FT-IR, while the matrices morphology by SEM. The influence of pH, CHXD concentration and GA as crosslinking agent for collagen on kinetics of CHXD delivery from the above matrices using a modified USP paddle method is also presented.

**RESULTS**

The superposed FT-IR spectra of the twelve collagen matrices are shown in Fig. 1.

The ratios of absorbencies of FT-IR bands $A_{III}/A_{1450}$ and the differences $(\nu_{AI} - \nu_{AII})$, cm$^{-1}$, used to evaluate the integrity of collagen triple helical structure, as well as of the ratios $A_{I}/A_{A}$ – useful to appreciate the extent of collagen crosslinking are given in Table 1.

The digital SEM microscopy images of the reference samples having the pHs 3.8, 7.4 and 7.4 with 0.15% GA (a-c), as well as of the corresponding ones containing 0.02 (d-f) and 0.05% CHXD (g-i) using a magnification of 200× are presented in Fig. 2a-i.

![Fig. 1 – Superposed FT-IR spectra of collagen matrices obtained from collagen hydrogels having pH 3.8 containing: 1 – 0, 2 – 0.02, 3 – 0.05 and 4 – 0.10% CHXD; pH 7.4 and: 5 – 0, 6 – 0.02, 7 – 0.05 and 8 – 0.10% CHXD; pH 7.4, 0.15% GA and: 9 – 0, 10 – 0.02, 11 – 0.05 and 12 – 0.10% CHXD.](image-url)
In vitro release of chlorhexidine digluconate

Table 1

The ratios A_{III}/A_{1450} and A_{II}/A_{X} and differences (ν_{A_{I}} - ν_{A_{II}}), cm\(^{-1}\), obtained from FT-IR spectra of collagen porous matrices in the absence and presence of CHXD or/and GA

<table>
<thead>
<tr>
<th>CHXD, %</th>
<th>pH</th>
<th>3.8</th>
<th></th>
<th>7.4</th>
<th></th>
<th>Crosslinked</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A_{III}/A_{1450}</td>
<td>A_{II}/A_{X}</td>
<td>(ν_{A_{I}} - ν_{A_{II}}), cm(^{-1})</td>
<td>A_{III}/A_{1450}</td>
<td>A_{II}/A_{X}</td>
<td>(ν_{A_{I}} - ν_{A_{II}}), cm(^{-1})</td>
<td>A_{III}/A_{1450}</td>
<td>A_{II}/A_{X}</td>
<td>(ν_{A_{I}} - ν_{A_{II}}), cm(^{-1})</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.98</td>
<td>1.27</td>
<td>97</td>
<td>2.00</td>
<td>1.78</td>
<td>100</td>
<td>2.48</td>
<td>3.40</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>2.00</td>
<td>1.36</td>
<td>94</td>
<td>2.93</td>
<td>1.68</td>
<td>100</td>
<td>2.53</td>
<td>1.70</td>
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</tr>
<tr>
<td>0.05</td>
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<td>1.36</td>
<td>97</td>
<td>3.54</td>
<td>1.69</td>
<td>100</td>
<td>3.60</td>
<td>1.29</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>2.15</td>
<td>1.37</td>
<td>94</td>
<td>4.86</td>
<td>1.48</td>
<td>100</td>
<td>5.11</td>
<td>1.16</td>
<td>100</td>
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</tr>
</tbody>
</table>

Fig. 2 – SEM images 200× for reference matrices with pH: a – 3.8, b – 7.4, 7.4 and c – 0.15% GA; d-f – the same matrices containing 0.02% CHXD and g-i – 0.05% CHXD.
Precipitation of CHXD within the matrices having slight basic pH and 0.1% CHXD, regardless of the presence of GA, can be seen in Fig. 3a,b, in which their SEM images are presented.

The curves representing the cumulative release of CHXD from the three series of collagen porous matrices are shown in Fig. 4.

DISCUSSION

FT-IR can provide unique information on the molecular structure of proteins and has been intensively used for the structural analysis of collagen. Many of the vibrational bands characteristic of peptide groups and side chains provide information on protein secondary and tertiary structures. That is why collagen is usually analyzed into the frequencies range 1800-100 cm\(^{-1}\) in which the bands amide I, II and III are found.

Fig. 4 – Cumulative release of CHXD from collagen matrices containing: 2×10\(^{-2}\) (pHs: B – 3.8, C – 7.4, D – 7.4 and 0.15% GA); 5×10\(^{-2}\) (E-G – the same order of curves); 10\(^{-1}\)% (H-J– the same order of curves) CHXD.

The most important band for studying the higher order structures in collagen is amide I, \(A_\alpha\), at about 1650 cm\(^{-1}\), which is especially sensitive to secondary structures. Its deconvolution shown that it consists of nine bands assigned to: 1 for ester group, 6 characteristic to secondary structure of collagen (1 for anti-parallel \(\beta\)-strand, 2 for \(\beta\)-turn, 1 for \(\alpha\)-helix), 1 assigned to unordered structure and 1 corresponding to the triple helix, which absorbs at 1638 cm\(^{-1}\). The most intense is the band assigned
to $\alpha$-helix, which absorbs at 1656 cm$^{-1}$, so such structures do not exist as such in type I collagen.\textsuperscript{32}

Involved with triple helical structure of collagen is the band amide III,\textsuperscript{38,39} $\nu_{\text{II}}$, found around 1550 cm$^{-1}$, which consists of one amine band at 1592 cm$^{-1}$, three amide II bands (1571, 1554 and 1546 cm$^{-1}$, the last having the highest intensity) and one assigned to tyrosine cycle.\textsuperscript{32}

The superposed FT-IR spectra of collagen matrices from Fig. 1 show that all the absorbencies of bands are pH dependent: the matrices obtained from acid hydrogels give more intense absorption bands both in the absence and presence of CHXD, while those obtained from slight basic hydrogels are pretty weak, irrespective of the presence of GA, part of them overlapping over some spectral regions. The most intense bands were obtained for the reference matrix resulted from the acid hydrogel regardless the frequency.

All the FT-IR bands related to the triple helical structure of collagen and crosslinking are found in the matrices at practically the same frequencies as in the collagen hydrogels from which they were prepared:\textsuperscript{30} amide I – 1639-1637 cm$^{-1}$ irrespective of pH, CHXD presence and concentration and GA, amide II – 1543-1539 cm$^{-1}$ in matrices obtained from acid hydrogels and 1539 cm$^{-1}$ in all the other matrices, amide III – 1238-1234 cm$^{-1}$, amide A – 3290-3294 cm$^{-1}$. It must be remarked that the differences do not exceed 4 cm$^{-1}$, the spectral interval between data points. These demonstrate that freeze-drying does not disturb the overall collagen organization.

The ratios $\nu_{\text{II}}/\nu_{\text{A1450}}$, that measures the triple helical conformation of collagen molecules from fibrils,\textsuperscript{39} are all higher than unity, as Table 1 shows, which proves the preservation of conformation of collagen molecules into fibrils. The values of this ratio increase slightly with CHXD concentration for matrices obtained from acid hydrogels. In the case of those obtained from slight basic hydrogels these ratios are higher and increase more with CHXD concentration. The situation is the same if GA is present, but the values are slightly higher.

The values for the difference $(\nu_{\text{AI}} – \nu_{\text{AI}})$, used to emphasize the presence of denatured collagen (differences higher than 100 cm$^{-1}$ indicate denatured collagen), range between 94 and 97 cm$^{-1}$ for the matrices obtained from the hydrogels having the pH 3.8 and have the value 100 cm$^{-1}$ for all the other matrices. Thus, both the values of $\nu_{\text{II}}/\nu_{\text{A1450}}$ and $(\nu_{\text{AI}} – \nu_{\text{AI}})$ demonstrate that the triple helical conformation is not disturbed in collagen matrices by freezing, pH and CHXD. The same is valid for GA in the absence or presence of CHXD.

The quantity able to measure the extent of crosslinking into a collagenic material is the ratio $\nu_{\text{AI}}/\nu_{\text{AA}}$, values also given in Table 1: the higher the ratio the most extended the crosslinking.\textsuperscript{30}

The $\nu_{\text{AI}}/\nu_{\text{AA}}$ values are smaller for the matrices obtained from acid collagen hydrogels and increase gentle when CHXD is added, from 1.27 to 1.37, regardless the CHXD concentration. This may indicates a very slight crosslinking effect of CHXD, produced by the binding of its amine groups to the very few carboxyl groups existing in collagen at pH 3.8. In the case the hydrogels from which the matrices were obtained were slight basic, the values of the ratio $\nu_{\text{AI}}/\nu_{\text{AA}}$ were higher and decreased slightly (from 1.78 to 1.48) when CHXD concentration increased. This can be explained by the CHXD binding to the carboxyl groups of collagen present in large amounts at this pH, which destroys a part of hydrogen bonding between the collagen fibrils that assure gel consistency. The larger the amount of CHXD the higher the hydrogen bonding destruction. Thus, small regions consisting of collagen fibrils crosslinked by CHXD may be formed, which produce a slight discontinuity of hydrogel that must be reflected also in the matrices morphology, results in agreement with CD spectra and rheological data obtained in a previous paper\textsuperscript{30} for the hydrogels from which the matrices were obtained. The highest $\nu_{\text{II}}/\nu_{\text{AA}}$ value was obtained for the reference sample containing GA, as expected, GA functioning as crosslinking agent for collagen in slight basic medium.\textsuperscript{41} It is expected that GA reacts more quickly with amine groups of CHXD than with those of collagen (crosslinking time 24 h at 4°C) and the added CHXD consumes GA. GA being in low amount (the lowest CHXD/GA molar ratio is 1.37 and the highest 6.85), the excess CHXD has the same effect as in the absence of GA: it crosslinks collagen, producing regions consisting of collagen fibrils crosslinked by CHXD, which results in a slight phase separation that can be also visually observed.

The size and morphology of the pores in the collagen-based biomaterials normally make a great contribution to their ability to facilitate tissue regeneration.

Taking account of the influence of freezing temperature on pore size and form,\textsuperscript{9} pretty large
and relatively inhomogeneous interconnected pores are expected for the prepared matrices, the freezing temperature being (–40)°C. Moreover the matrices obtained from acid hydrogels must have a fibrous structure, a large number of channels and smaller pore size compared to those resulted from slight basic ones. The morphology of matrices also has to reflect the interactions taking place between collagen and CHXD and/or GA in the corresponding hydrogels from which they were obtained. SEM measuring both the matrices’ pore size and the extent of cross linking, it was used to emphasize their morphology as shown in Fig. 3a-i.

Figure 3a shows that the reference matrix obtained from acid collagen hydrogel has a lattice-like lamellar structure with lamellar distances varying between 50 and 160µm, connected by fibrils and fibres which form more or less distorted annular pores. If the pH of the hydrogel was 7.4 – Fig. 3b – both irregular and ring-shaped pores of different size, interconnected by larger number of fibrils, fibres and fibre agglomerates can be seen. The presence of GA into the collagen hydrogel gives a matrix with the same aspect as the previous one, but more agglomerates can be observed (Fig. 3c), which are due to the collagen crosslinking.

Introduction of 0.02% CHXD into the collagen hydrogel with pH 3.8 preserves the matrices lamellar structure as Fig. 3d shows, but the lamellae are more different in thickness and more closed – the distances varies between 40 and 110µm – due to the slight cross-linking effect of CHXD on collagen, emphasized also by FT-IR. When the concentration of CHXD increases at 0.05% (Fig. 3g) the lamellae are even more closed and irregular.

The presence of GA in CHXD containing collagen hydrogels gives matrices with higher amounts of agglomerates, their size increasing with CHXD concentration as Figures 3f and 1 show, almost compact regions appearing in the last Figure, in which a low number of small near spherical CHXD particles can be also seen.

The separation of CHXD is more evident in the two matrices obtained from slight basic hydrogels, shown in Figure 4a,b, for which a magnification of 600x was used. They have spherical shapes, with diameters ranging between 2 and 6 µm. Their presence can be explained by the regression of CHXD solubility at basic pH.

Drug release of CHXD contained into the collagen matrices presents the same characteristics as in the case of the corresponding hydrogels: depends on the pH of the hydrogels from which they were obtained, the lowest amounts being released by the matrices obtained from the acid hydrogels and the highest ones from the non-crosslinked ones obtained from hydrogels with slight basic pH containing 0.1% CHXD; increases with CHXD concentration irrespective of pH and presence of GA; GA reduces the amount of CHXD released regardless the amount of CHXD.

**EXPERIMENTAL**

**Preparation of collagen matrices.** The collagen hydrogels having the composition shown in Table 1 and prepared as described in a previous paper were freeze-dried for 3½ h at (–40)°C in the lyophizer already refrigerated at (–40)°C and then subjected to lyophilisation at the constant pressure of 0.12 atm in the following conditions: (–40)°C for 5 h, 10°C for 10 h, 20°C for 8 h, 35°C for 13 h and 40°C for 7 h using a Delta 2-24 LSC (Martin Christ, Germany) lyophiliser. Considering also the time required for lyophiliser refrigeration, the whole process lasted for 48 h.

**FT-IR spectra** were obtained using an ABB MB3000 MID-IR spectrometer equipped with a DTGS detector and Horizon software. Data were acquired by ATR technique using a PIKE 45 degree ZnSe trough plate with volatile cover Horizontal ATR. The spectra were corrected for ATR effect and then transformed into absorption ones. Each spectrum is the average of 32 scans, with a spectral interval between data points of 4 cm⁻¹.

**SEM** was performed with a VEGA II LMU device equipped with a LVSTD type detector using the following parameters: accelerating voltage – 30 kV, emission current – 77 µA, system pressure 10 mPa, pixel size X/Y – 1.47 µm, dwell time – 437 ms, spot size – 48 nm; magnification – 200x and 600x.

**In vitro release of chlorhexidine digluconate** was determined in triplicate at 37±0.1°C using a modified “sandwich” device (USP paddle method) and phosphate buffer with pH 7.4 as release medium. Aliquots of 5 mL were withdrawn from the release medium at different times and completed with the same volume of fresh buffer (of 37°C). Concentration of CHXD was measured at 255 nm, and the cumulative amounts of CHXD released were determined using the calibration curve.

**CONCLUSIONS**

FT-IR spectra of collagen matrices show that the triple helical structure of collagen from the hydrogels having the pHs 3.8 and 7.4 was preserved during the freeze-drying process both in the absence and the presence of 0.02, 0.05 and 0.10% CHXD and/or GA.

Matrices obtained from acid hydrogels have lattice-like lamellar structure that becomes denser with increasing of CHXD concentration due to its crosslinking effect. This is absent in the matrices obtained from slight basic hydrogels, both in the
absence and the presence of GA, in which some fiber agglomerations can be seen. The agglomeration increases with CHXD concentration and becomes significant in the matrices containing GA, especially when CHXD concentration is 0.05%. In the matrices containing 0.1% CHXD a part of it separates due to the regression of its solubility in basic media.

The release of CHXD from the collagen matrices presents the same characteristics as in the case of the hydrogels from which they were obtained: increase with CHXD concentration irrespective of pH and presence of GA, the lowest amounts of CHXD are released from the matrices obtained from the hydrogels with acid medium and the highest from the non-cross-linked matrices obtained: increase with CHXD concentration in the matrices from which they were case of the hydrogels from which they were

REFERENCES
