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In Vitro Biocompatibility of Human Endothelial Cells with Collagen-Doxycycline Matrices

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The aim of this study was to test the biocompatibility between collagen-doxycycline matrices with various porosities and human endothelial cells. Collagen matrices were prepared by freeze-drying process. The crosslinking degree of collagen matrices was evaluated by FT-IR spectroscopy, the matrices morphology by SEM microscopy. The biocompatibility of collagen matrices with endothelial cells was monitored by fluorescence and transmission electron microscopy. We found that the three-dimensional structures of matrix with 1.2% collagen, 0.2% doxycycline crosslinked with 0.25% glutaraldehyde was optimal in terms of biodegradability, morphology and endothelial cells biocompatibility indicating the use of these scaffolds in tissue engineering.

Keywords Biocompatibility; collagen-doxycycline matrices; endothelial cells

Introduction

Tissue engineering is a rapidly evolving multidisciplinary field that applies the principles of biology and engineering in order to develop tissue substitutes to restore, repair or improve the function of diseased or damaged human tissues [1]. Cell based therapies might therefore offer hope for a number of diseases, in particular those in which, single pharmacological agents are not sufficient. The success of numerous therapies in regenerative medicine requires the ability to control the formation of stable vascular networks within tissues. The formation of new blood vessels, or neovascularization, is mediated, in part, by the interaction between endothelial cells and insoluble factors of the extracellular microenvironment. These interactions are determined by the chemical, physical, and mechanical properties of the matrix [2].

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In tissue engineering, the role of the scaffold is comparable to the role of the extracellular matrix and consists in supporting the development of cells and tissue [3]. Collagen is a significant constituent of the natural extracellular matrix (ECM) and plays an important role in the formation of tissues and organs, being involved in the functional expressions of cells.

Collagen scaffolds have been used in a variety of applications due to a number of valuable characteristics like low antigenicity, high biocompatibility and hemostatic and cell-binding properties [4]. It is now evident that collagen and collagen-derived fragments control many cellular functions, including cell shape and differentiation [5,6], migration [7], and synthesis of numerous proteins [8]. Several *in vitro* studies of cell-scaffold interactions and tissue synthesis as well as *in vivo* studies on induced tissue and organ revealed the excellent biological performances of collagen [9].

To promote cell adhesion and growth, a biologically active scaffold, must satisfy a number of features. The scaffold biomaterial has to be biocompatible and degrade in the body at a controlled rate; the average pore diameter must be large enough for cells to migrate through the pores and small enough to provide a critical total surface area for appropriate cell binding and has to preclude the risk of infection during applications.

An essential element in graft procedure is the blood supply. The prevention of implant failure caused by hypoxia and following infection is still a challenge. However, in general, cell-based tissue engineering provides a successful treatment in wound healing disorders.

It is known that endothelial cells that form the inner lining of blood vessels, participate in important physiological processes including exchanges of molecules, coagulation and wound healing. Also these cells are essential for vascularisation of the new tissue during the wound healing and tissue formation processes. A requirement for promoting faster vascularisation is the presence of large pore sizes into the scaffolds [10]. The scaffolds made up of porous collagen matrices provide a three-dimensional (3D) structure which has a significant effect on cellular activity [4].

Three-dimensional cell culture systems offer a milieu to study biosecretory, migratory, and proliferative functionality [11–14]. Embedding of endothelial cells in three-dimensional collagen-based matrices allows them to grow and attain confluence in a controlled environment. Such constructs permit endothelial cells to retain a quiescent state, the secretion of essential regulatory factors and the associated potential for vaso-regulatory control, within matrices (vehicles) that can be stored, manipulated, functionally validated, and implanted at will at sites protected from environmental forces [15–18].

In this study we investigated the biocompatibility of collagen matrices with different degrees of crosslinking and antibiotic content (doxycycline) with endothelial cells in order to select the proper matrix for best development of these cells.

Materials and Methods

Collagen Matrices Preparation

Type I fibrillar collagen gel having a concentration of 2.67% (w/w) was extracted from calf hide as previously described [3]. Doxycycline hyclate (DH) was purchased from Sigma-Aldrich and glutaraldehyde (GA) was obtained from Merck (Germany).

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

Sodium hydroxide and phosphate buffer solution (PBS), pH, 7.4 were of analytical grade.

The concentration of each collagen gel was adjusted at 1.2%. DH in concentration of 0.2% reported to the dry substance was added into collagen gels and the pH adjusted at 7.4 with 1 M sodium hydroxide (under stirring). The crosslinking of collagen-doxycycline gels was achieved employing different concentration of GA (0, 0.15, 0.20, 0.25 and 0.30%) reported to the weight of dry collagen at 4°C for 24 hours. The crosslinked gels were cast in glass dishes of 14.7 cm diameter and 1 cm height at 20°C and freeze-dried as follows: cooling to −60°C (2 h), keep for 4 h, then heating with 5°C/hour (14 h) at 0.12 mbar and then heating (4 h) to 30°C at 0.01 mbar using the Christ Model Delta 2–24 LSC freeze-dryer, Germany. The composition and the codes of the matrices obtained by freeze-drying are given in Table 1.

Spectral characteristics of the matrices were determined using a FT-IR 6000 spectrophotometer with ATR reflection system MKII Golden Gate Single (Jasco) in IR (MID and NIR) region.

Determination of Water Uptake Capacity of Collagen Matrices

The water uptake capacity was carried out using phosphate buffer pH 7.4 as immersion medium (n = 3). Pieces of collagen matrices of ~2 cm^2 area were weighed (W_d) and then immersed in phosphate buffer pH 7.4. At established time intervals, the hydrated matrices were weighed (W_w) and water uptake was calculated using the following equation [19]:

$$Water\ uptake = \frac{W_w - W_d}{W_d}\ \text{g/g} \tag{1}$$

where W_w represents the weight of wet matrices at immersion time t and W_d denotes the weight of dry matrices.

Enzymatic Degradation

The enzymatic degradation was carried out with type I collagenase (Sigma-Aldrich, Germany) obtained from *Clostridium histolyticum* in physiological conditions (PBS, pH 7.4, 37°C). About 1 g of prepared matrix was incubated in 0.5 mL collagenase solution at 37°C for 36 h. The degradation reaction was stopped by cooling the samples at 0°C, then centrifuged (15 min) and the supernatant was freeze-dried and reported to the initial weight of the sample (g/g).

Scanning Electron Microscopy

The morphology of the collagen matrices was examined by scanning electron microscopy (SEM) using a Philips XL-30-ESEM TMP apparatus at an accelerating voltage of 25 kV.

Collagen-Doxycycline Matrices Colonization

For *in vitro* colonization, the human endothelial cell line, EA hy 926 (human aortic endothelial cells) were grown in DMEM culture medium containing 4,5% glucose, 10% fetal bovine serum, 20 μg/l sodium selenite, 30 mg/l ascorbic acid, and antibiotics (100 U/l penicillin, 100 U/l streptomycin, 50 U/l neomycin). Collagen-doxycycline matrices were sterilized for 24 hours with 70% ethanol, and then conditioned in the same culture medium for 24 hours and inoculated with endothelial cells (50.000 cells/ml). The cells on matrices were maintained in culture at 37°C in incubators with 5% CO_2 in air (v/v) and relative humidity over 95%. All experiments were done after 1 week of culture. Chemicals used for cell cultures were obtained from Sigma (USA) and tissue culture plates from Costar (Cambridge, MA, USA).

Hoechst Staining

After one week in culture, the cells on collagen matrices were washed in PBS, fixed in 2% paraformaldehyde (one hour) and then cryoprotected. After washing in phosphate buffer (PB: 0.2 M Na_2HPO_4, and 0.2 M NaH_2PO_4) ph 7.2, the specimens were kept in a solution of PB 0.1 M with 5% sucrose overnight at 4°C. Then, the probes were immersed in PB containing 5% glycerine for 15 minutes, PB with 10% glycerine for one hour, PB with 20% glycerine for 10 hours and PB with 50% glycerine for one hour at 4°C. 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 PBS for 15 minutes, stained with Hoechst 33258 (a specific DNA staining) for 15 minutes, washed in distilled water, mounted in glycerol and examined with a Nikon microscope equipped with epi-fluorescence and a filter G1-B; the micrographs were captured with a Sony DSC-S75 Digital Camera.

Endothelial Cell Viability

Cell viability was determined by MTT assay. Reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodium bromide (MTT) to a purple formazan reaction product by living cells – was used to estimate cell viability [20]. The cells cultured on collagen doxycycline matrices cubes (1 mm^3) were incubated with 0.5 mg mL^{-1} of MTT during the last 4 h of the culture period tested; the medium was then decanted, formazan salts were dissolved with 0.1 N HCl in anhydrous isopropanol and the absorbance was measured at 570 nm employing a TECAN 24-well plate reader. The results were expressed as viability percentage.

Transmission Electron Microscopy (TEM)

(TEM) was preformed according to Jinga *et al.* [21]. Throughout the procedure, all buffer solutions were used at pH 7.4 and the osmolality of 300 mOsm. Briefly, the cells grown on collagen supports were washed twice with 75 mM sodium cacodylate

buffer supplemented with 3% (w/v) sucrose (SCB) and fixed (90 min) in 2.5% glutaraldehyde in SCB and again washed twice with SCB. Then, the cultures were post-fixed in 2% (w/v) osmium tetraoxide in 150 mM sodium cacodylate buffer (CB) for 90 min, at 4°C, washed twice (2 min) in cacodylate buffer followed by mordanting in 1% (w/v) tannic acid (10 min) and two rinses in 1% (w/v) sodium sulphate in 100 mM cacodylate buffer [22]. The cultures were then rapidly dehydrated in increasing concentrations of ethanol: 70% (v/v) (5 min), 95% (5 min) and 100% (3 × 5 min) followed by Epon 812: ethanol 1:1 (v/v) for 30 min and then embedded in Epon 812 (100%). The resin was polymerized at room temperature for 1 hour, and then at 37°C for 4 hours and at 55°C for two days.

Thin sections obtained on a Reichert OmU3 ultramicrotome were stained with 7.5% (w/v) uranyl acetate (10 min) and with lead citrate 0.4% (w/v) for 1.5 min, and examined with a Philips EM 201C and Philips EM 400 (Philips, Holland) transmission electron microscopes. Chemicals used for electron microscopy were form Polysciences (USA), except sodium sulphate, lead citrate, and glutaraldehyde that were obtained from Merck (Germany), and tannic acid from Mallinckrodt.

Biomaterials colonization was monitored by fluorescence microscopy, transmission electron microscopy and viability by MTT assay.

Results and Discussions

Characterisation of Collagen Matrices

The collagen matrices were prepared by freeze-drying of collagen gels. Employing this technique, the ice crystal appeared within the gels forming a continuous, interpenetrating network of ice. The size of ice crystals depended on difference between the temperature of freezing and the actual temperature of material during the frozen process [23]. The structure of collagen scaffold was controlled by final temperature processes associated with freezing [4]. To obtain homogeneous scaffolds with uniform pore sizes we started to heat the samples from −60°C and kept a slow rate of heating of 5°C/hour during the freeze-drying process. To avoid denaturation of collagen, the final temperature of the freeze-drying was 30°C.

In general, to obtain collagen matrices with controlled biodegradability, improved biologically stability and good mechanical properties, the collagen gels are chemically crosslinked (i.e., with glutaraldehyde) [24]. The disadvantage of many biomaterials is the fact that they require chemical crosslinking of constituent polymers during the manufacture process, which can cause problems of toxicity due to the unreacted or partially reacted crosslinking agent [25].

Because of the high degradation rate of natural collagen *in vivo*, crosslinking *in vitro* is necessary to reduce biodegradation and maintain ability of fluid absorption of matrices used for tissue repairs. According to the requirements for specific tissue engineering applications, different concentrations of GA (0.15, 0.20, 0.25 and 0.30% respectively) were used to modulate the physico-chemical properties of collagen matrices. Depending on the concentration of collagen, doxycycline and glutaraldehyde, the matrices were name thus: M-B – the matrix which contain only type I collagen (1.2%), CD-B – the matrix with collagen (1.2%) and doxycycline (0.2%), CD-0.15, CD-0.20, CD-0,25 and CD-0,30 – the matrices which contain collagen (1.2%), doxycycline (0,2%) and that are crosslinked with glutaraldehyde (0.15, 0.20, 0,25 and 0,30% respectively).

FT-IR Spectra of Collagen Matrices

To determine if the triple helical structure of collagen was modified by exposure to doxycycline or glutaraldehyde the FT-IR spectra were registered. The FT-IR spectrum of control collagen matrix (M-B) is shown in Figure 1a. Absorption bands associated with the presence of amides were identified: amide A (A_A) at 3297 cm^{-1}, amide B at 2931 cm^{-1}, Amide I (A_I) – CO strength – at 1632 cm^{-1}, amide II (A_{II}) – NH band coupled with CN strength – at 1547 cm^{-1} and Amide III (A_{III})– NH band – at 1240 cm^{-1}. All these values are comparable with those previously reported [26].

The following semiquantitative relations determined the collagen structure modifications:

– A_{III}/A_{1450} ratio is correlated with maintaining of triple helical structure integrity; the ratio value has to be higher or equal with 1;
– A_I/A_A ratio is correlated with crosslinking degree: the higher A_I/A_A ratio the higher crosslinking degree.

Upon addition of doxycycline, the IR band characteristic for amide A shifted from 3297 to 3292 cm^{-1} (Fig. 1b) indicating the formation of strong hydrogen bonds between OH groups. Our results showed that in our experimental conditions, the doxycycline does not modify the structure of collagen since the A_{III}/A_{1450} ratio was 1.16 for CD-B. Crosslinking with GA results in A_{III}/A_{1450} ratio values close

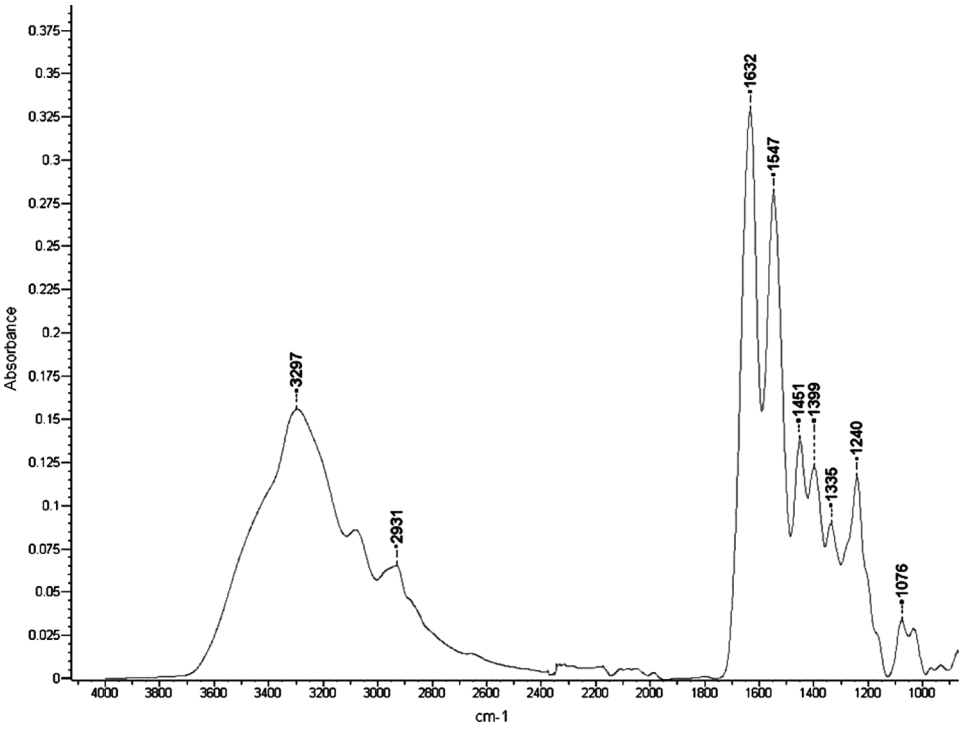


Figure 1. (a) – FT-IR spectrum of control collagen matrix **M-B**; (b) – FT-IR spectrum of CD-B matrix.

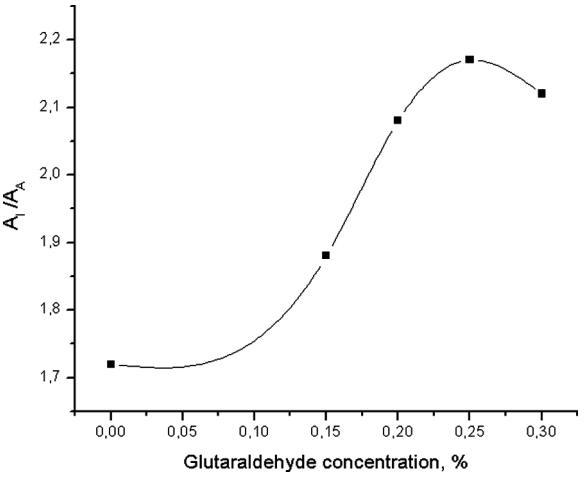


Figure 2. The ratio A_I/A_A as a function of glutaraldehyde concentration.

to 1, but lower than the uncrosslinked one (CD-B). This shows that GA interacts with lysine from side chains of collagen triple helix without any modification of tertiary structure.

In order to prove the crosslinking degree, the A_I/A_A ratio for samples with doxycycline were determined and their values are presented in Figure 2. This ratio increased from 1.72 (which corresponds to CD-B) to 2.17 – a maximum value obtained for matrix crosslinked with 0.25% GA (CD-0.25). The data showed that the use of higher concentration of GA decreased slowly the A_I/A_A ratio until 2.12, a value corresponding to CD-0.30 (Fig. 2).

The degree of matrix crosslinking increased up to 0.25% GA concentration, the value at which maximum crosslinking was attained; over this value, it is considered that the GA does not react with the collagen matrix (Fig. 2).

Water Uptake of Collagen Matrices

Collagen is a hydrophilic polymer. Its swelling, an important step in the degradation process, is determined by the water uptake and can be controlled by the degree of crosslinking. The liquid is absorbed by matrices, increasing the sample volume. For the prepared matrices employed in our experiments, the water uptake during 12 hours is presented in Figure 3.

We found that all matrices have a comparable profile of water uptake. However, comparing the water uptake for M-B and CD-B (Fig. 3a and b) we found that doxycycline increased the ability for water absorbtion of CD-B matrix, the amount of absorbed liquid being 1.7 times higher than the value obtained for M-B matrix during the 12 hours examined.

The water uptake assay showed that swelling of matrices was influenced by the degree of crosslinking: the higher crosslinking was associated with lower water uptake. The matrix crosslinked with 0.25% GA exhibited lower swelling than that crosslinked with 0.30%. These data corroborate well with the results of FT-IR spectra demonstrating that matrix CD-0.25 has the higher degree of crosslinking.

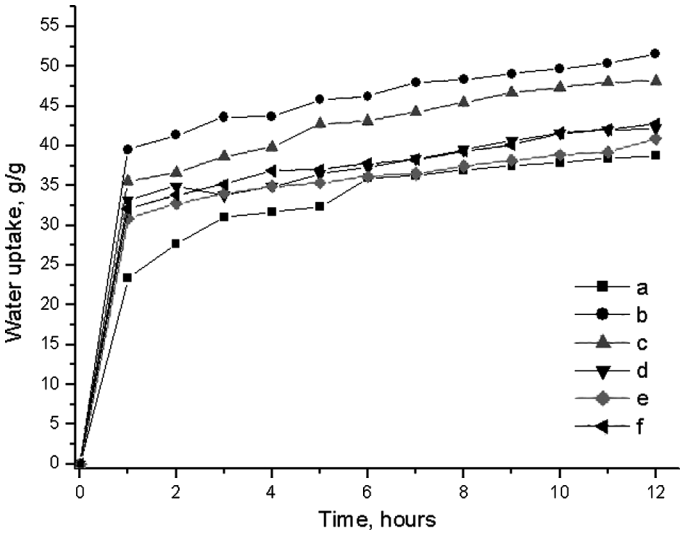


Figure 3. Water uptake for collagen matrices: (a) – M-B; (b) – **CD-B**; (c) – CD-0.15; (d) – CD-0.20; (e) – CD-0.25; and (f) – CD-0.30.

Enzymatic Degradation of Collagen Matrices

Another important aspect is the rate of degradation of collagen matrices, because *in vivo* resorption influences the ability of tissue regeneration. The digestion with collagenase reveals indirectly the degree of crosslinking of collagen.

In our experiments, we evaluated the residual amount of collagen after 36 hours of incubation of matrices with collagenase (Fig. 4). As seen in Figure 4, after 36 hours

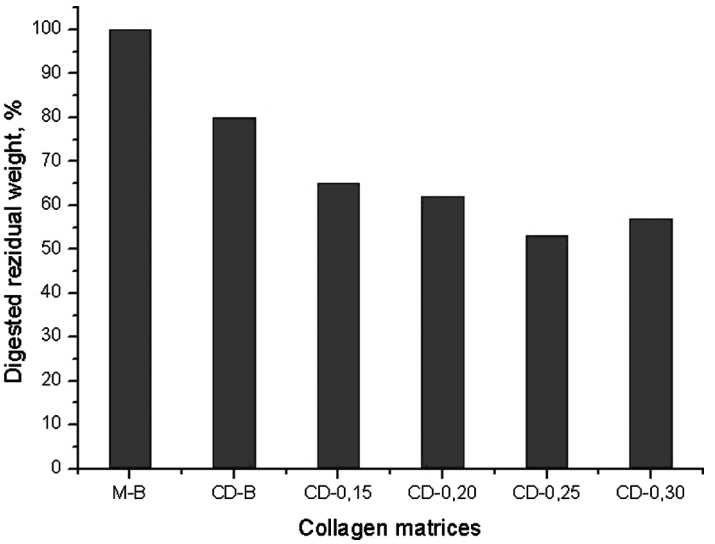


Figure 4. Resistance to collagenase of collagen matrices estimated by the residual digested collagen.

of incubation with collagenase, the control collagen matrix (M-B) was completely digested, whereas the collagen with doxycycline, the uncrosslinked samples (CD-B) were digested by ~80% at the same time interval, because the doxycycline inhibit the action of collagenase [27]. All crosslinked matrices had slightly higher resistance compared to the uncrosslinked samples under the same treatment conditions. Crosslinking with GA reduced biodegradability resulted in slower degradation; the CD-0.25 matrix had the lowest rate on biodegradation. After 48 hours, the degradation was completed for all matrices; as a result, a multitude of precipitated collagen fibers was detected in the collagenase solution.

Collagen matrices are porous structures with pore sizes ranging between 50 and 1500 μm [28]. Depending on the form, orientation and size of the pores the structures are dense or rarefied.

Scanning Electron Microscopy of Collagen Matrices

The effect of collagen crosslinking and its interaction with doxycycline on matrix morphology and the measurement of pore sizes was investigated by scanning electron microscopy.

The M-B matrix appeared as a uniform porous structure with regular pores interconnected through a multitude of collagen fibers (Fig. 5). The measured pore diameters varied between 70 and 290 μm.

Although in the literature it is specified that, in general drugs do not influence the porous structure of collagen [29], we have found that doxycycline produced more porous structures (Fig. 6) and the CD-B presents large pores with diameters between 280 and 400 μm (Fig. 6a). Interestingly, as revealed by SEM examination, the crosslinked matrices (exposed to GA) exhibited much smaller pores than the uncrosslinked one (200 – 360 μm) (Fig. 6b). This difference may be due to the crosslinking agent reaction.

The CD-0.25 matrix has the densest structure (Fig. 6b) as a result of a maximum crosslinking.

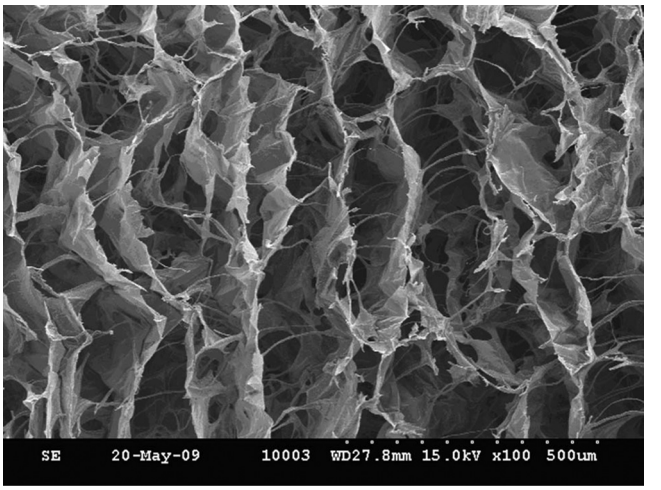
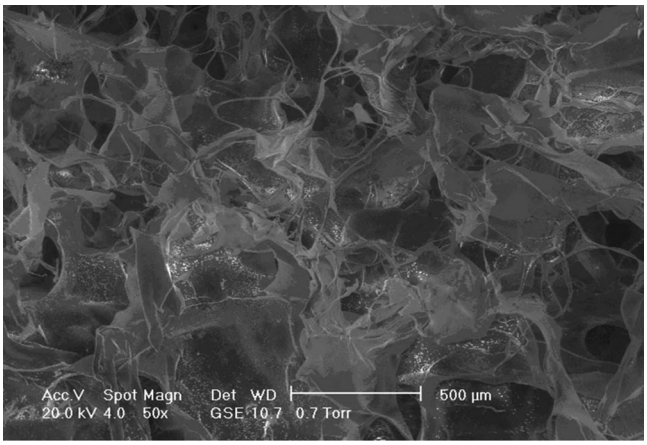
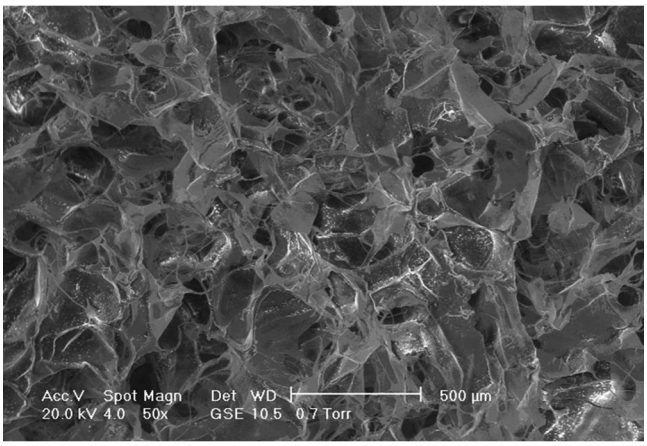


Figure 5. M-B matrix morphology analyzed by scanning electron microscopy.



(a)



(b)

Figure 6. CD-B – (a) and CD-0.25 – (b) matrices as seen by SEM examination.

The results obtained by FT-IR analysis, water uptake and enzymatic degradation are in close correlation with SEM morphology of matrices. The crosslinking degree is more evident at 0.25% GA treatment of matrix. Above this concentration the GA remained active in collagen matrix. As already reported the biomaterials for tissue engineering have to be resistant of degradation and to be proper for cells attachment, migration and proliferation [30].

Endothelial Cell Colonization on Collagen-Doxycycline Matrices

The experiments done for *in vitro* biocompatibility evaluation of prepared matrices with endothelial cells showed that the uncrosslinked collagen matrices (M-B and CD-B) dissolved in the culture medium. In contrast, the crosslinked matrices (with GA) maintained their integrity in the culture medium. For this reason, the biocompatibility with endothelial cells was studied only on crosslinked collagen matrices.

Employing Hoechst staining, we have found that crosslinked collagen matrices sustained cellular growth (Fig. 7); no significant differences were observed between the endothelial cells grown on these matrices. All crosslinked collagen-doxycycline matrices allowed three-dimensional growth (at the surface and inside of the matrices) within the structural pores (Fig. 7).

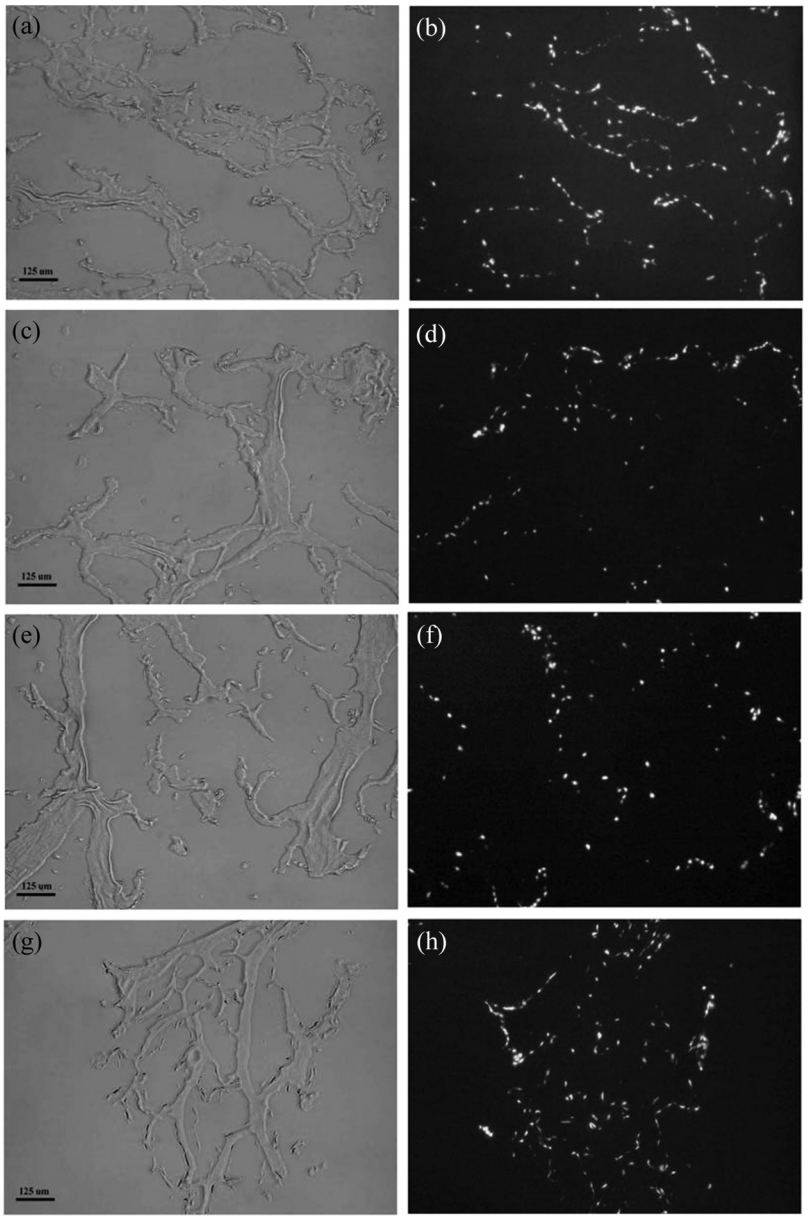


Figure 7. Endothelial cells cultured on collagen matrices: left panels: phase contrast microscopy; right panels: Hoechst staining. (a, b) – CD-0.15; (c, d) – CD-0.20; (e, f) – CD-0.25; and (g, h) – CD-0.30.

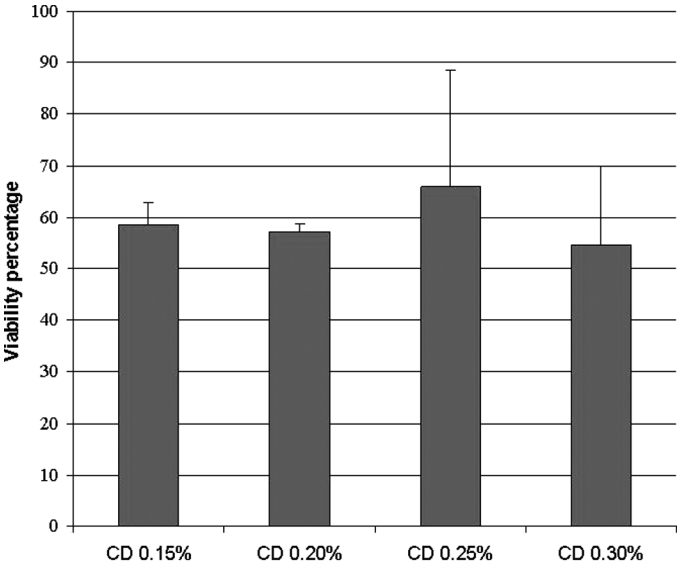


Figure 8. Endothelial cell viability on collagen matrices crosslinked with various concentration of GA.

Endothelial Cell Viability on Collagen-Doxycycline Matrices

Figure 8 shows the viability of endothelial cells cultured on collagen-doxycycline matrices crosslinked with different concentrations of glutaraldehyde and evaluated by MTT reduction. MTT is metabolized to a purple formazan salt by mitochondrial enzymes in living cells and the absorbance is proportional to the number of viable cells.

Comparing the cells grown on different collagen-doxycycline matrices, we found no statistically significant differences between endothelial cultured cells. However, the higher percent of viability was registered for CD-0.25 (65%) and the lower for CD-0.30. This indicates that collagen-doxycycline matrices crosslinked with glutaraldehyde allow endothelial cells colonization, adhererence and the cells maintain their viability.

Ultrastructure of Endothelial Cells Grown on Collagen Matrices

Cellular ingrowth within a sponge largely depends on the porosity and on the presence of a fibrous structure. The structure of collagen matrices that we have obtained exhibits interconnected holes that allow endothelial cell migration. Because the pore sizes of matrices varies between 70 and 400 μm, the endothelial cells that have 8–12 μm in diameter are not restricted to penetrate into matrix pores. Moreover, within the crosslinked collagen-doxycycline matrices, the endothelial cells maintain their normal structure as shown in Figure 9.

The ultrastructural examination of the endothelial cells integrated in the biocompatible collagen supports revealed the presence of a well developed rough endoplasmic reticulum, and numerous mitochondria (Fig. 9a,b,c). The cells penetrated the scaffolds pores and integrated in their structure (Fig. 9c,d).

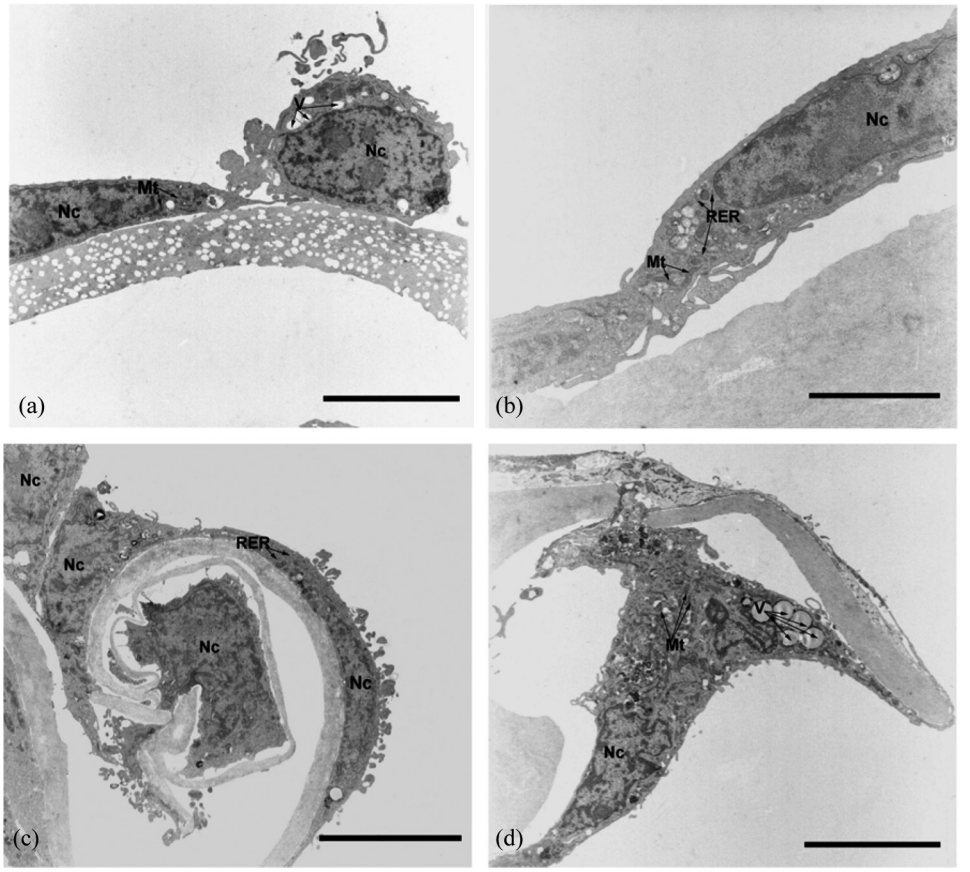


Figure 9. Ultrastructure of endothelial cells grown on collagen matrices: (a) – CD-0.15; (b) – CD-0.20; (c) – CD-0.25; (d) – CD-0,30; Nc – nucleu, V – vacuoles, RER – rough endoplasmatic reticulum, Mt – mitochondria; Bars: (a) 3,92 μm; (b) 2.38 μm; (c) 1.66 μm; (d) 3.92 μm.

However, some cells grown on collagen-doxycycline scaffolds crosslinked with 0.30% glutaraldehyde presented many large vacuoles with an electron-dense content, indicating that these matrices affected the integrity of endothelial cells (Fig. 9d). This may be due to the excess of unreacted GA that could be toxic to the cells. Nevertheless, the results indicate that collagen scaffolds can be successfully employed in wound healing and tissue engineering.

Conclusions

Various degrees of crosslinking and porosities of collagen matrices were obtained employing different concentrations of glutaraldehyde. FT-IR analysis showed that collagen kept its triple helical structure when reacted with doxycycline or upon crosslinking with glutaraldehyde. In addition, the FT-IR, water uptake and enzymatic degradation experiments showed that collagen matrices degradation depends on the crosslinking degree. Maximum crosslinking was obtained when 0.25% glutaraldehyde was used. By comparison, at this concentration, we obtained the lower water

uptake, a minimum biodegradation and the densest porous matrix with smallest pores. Although all crosslinked collagen-doxycycline matrices are biocompatible with endothelial cells, the best viability was registered for matrix crosslinked with 0.25% glutaraldehyde. Transmission electron microscopy shows that endothelial cells populated and developed best on CD-0.25 matrix. Furthermore, concentrations over 0.25% glutaraldehyde in collagen matrices became toxic to the cells as revealed by the appearance of many vacuoles with electrono-dense content in endothelial cells grown on CD-0.30 matrix. In conclusion, the three-dimensional structure of matrix with 1.2% collagen, 0.2% doxycycline crosslinked with 0.25% glutaraldehyde appear to be optimal in terms of biodegradability, morphology and endothelial cells biocompatibility.

Acknowledgments

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