

COLLAGEN-DOXYCYCLINE MATRICES USED IN TISSUE ENGINEERING

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Abstract

Porous collagen matrices with defined physical, chemical and biological characteristics are interesting biomaterials for tissue engineering and drug delivery systems. In this study collagen hydrogels with the same content of doxycycline and cross-linked with different concentrations of tannic acid (0, 5, 10 and 20%) were obtained. They were rheologically characterized and dried by lyophilization in order to obtain matrices (spongy forms). The obtained matrices were analyzed by scanning electron microscopy (SEM). Their biocompatibility with endothelial cells was monitored by fluorescence and transmission electron microscopy (TEM) and their viability was assessed by MTT assay. The results showed that matrices allow endothelial cells to develop normally, representing promising supports both for drug delivery systems and tissue engineering.

Rezumat

Matricile poroase de collagen cu proprietăți fizice, chimice și biologice bine definite reprezintă biomateriale interesante pentru ingineria tisulară și pentru sistemele de cedare a medicamentelor. În această lucrare s-au obținut hidrogeluri de collagen cu același conținut de doxiciclină și reticulate cu diferite concentrații de acid tanic (0, 5, 10 and 20%). Acestea au fost caracterizate reologic și liofilizate în vederea obținerii de matrici poroase. Matricile obținute au fost analizate prin SEM (microscopie de baleiaj electronic). Biocompatibilitatea cu celulele endoteliale a fost monitorizată prin microscopie de fluorescență și microscopie electronică de transmisie (TEM), iar viabilitatea prin tehnica MTT. Rezultatele au arătat că aceste matrici permit dezvoltarea normală a celulelor endoteliale, reprezentând suporturi promițătoare pentru sistemele de cedare a medicamentului și ingineria tisulară.

Keywords: collagen, endothelial cells, biocompatibility

Introduction

Therapeutic approaches that promote wound healing after burns represent an important field of tissue engineering. Attention has been

directed to the administration of controlled drug delivery systems based on collagen in combination with some antiseptic substances that can prevent infections and promote tissue recovery.

As a natural biopolymer, collagen provides biologically specific signals for molecular interaction with the delivered cells and interact specifically with cells of the target tissue [1- 4].

Few controlled drug delivery systems based on collagen were clinically tested and introduced on the medical market, although they have been intensely researched all over the world. The future of drug delivery systems focuses on the so called smart systems, able to maintain or control the amount of released drug [5, 6].

Engineering skin substitutes by tissue engineering approaches has relied upon the creation of three dimensional scaffolds as extracellular matrix (ECM) analogue to guide cell adhesion, growth, and differentiation to form skin functional and structural tissue. The three dimensional scaffolds with antiseptic properties can not only cover wound and give a physical barrier against external infection as wound dressing, but also can provide support both for skin cells and prevent infection extending. Thus, the administration of three dimensional collagen matrices with antiseptic properties represents a strategy in wound healing treatment.

The objective of this study was the preparation and characterization of matrices (spongy forms) which consist in type I collagen in combination with two antimicrobial drugs: doxycycline hyclate – an antibiotic used especially for the treatment of periodontic disease and tannic acid – an antiseptic for wound treatment. These two drugs have antiseptic properties; more, the tannic acid represents a unique combination of anti-microbial, anti-inflammatory and anti-oxidant action [7-11]. Tannic acid, in various concentrations, is also a cross-linking agent, able to control the release of doxycycline from the collagen-based delivery system [12].

Collagen-doxycycline and collagen-doxycycline-tannic acid delivery systems in the form of matrices were not studied so far. Obtaining these systems is an innovation in the controlled drug delivery field both on national and international level.

Materials and Methods

Type I collagen was obtained from calf hide by chemical treatment (acid and basic) using the technology previously described [13]. Doxycycline hyclate was purchased from Fluka, BioChemica, China, tannic acid (TA) from Sigma-Aldrich, Germany and glutaraldehyde (25% aqueous solution) from Merck, Germany.

Collagen hydrogel based on 1.2% collagen and 0.2% doxycycline hyclate (reported to the collagen gel) was obtained at 3.8 pH. Then the hydrogels were crosslinked with different amounts of tannic acid (0, 5, 10 and 20% reported to the dry collagen).

Rheological measurements for hydrogels were performed using a rotational viscometer Multi-visc Rheometer-Fungilab equipped with standard spindles and an ultrathermostat ThermoHaake P5.

Collagen matrices (spongiuous form) were obtained by freeze-drying (lyophilizing) of the hydrogels using the Delta 2-24 LSC Christ lyophilizer, Germany and they were named as shown in Table I.

Table I
Codification of the matrices

Collagen matrix	M-A	CD-A	CD-T5	CD-T10	CD-T20
Doxycycline, %	0	0.2	0.2	0.2	0.2
Tannic acid, %*	0	0	5	10	20

The **scanning electron microscopy** images were registered using a Hitachi S-2600N scanning electron microscope with EDX spindle and a resolution of up to 4 nm and a 15 – 300 x magnification.

For ***in vitro* colonization** we used the human endothelial cell line EA hy 926 (human aortic endothelial cells) grown in Dulbecco's Modified Eagle Medium (DMEM) 4.5‰ glucose medium supplemented with 10% fetal bovine serum and antibiotics (100U/L penicillin, 100U/L streptomycin, 50U/l neomycin). Collagen matrices were sterilized with 70% ethanol and conditioned in the same culture medium and then inoculated with endothelial cells (50,000 cells/mL). Cells were maintained in culture at 37°C in incubators with 5% CO₂ in air (v/v), and relative humidity over 95%. All experiments were performed after one week of culture. *Chemicals* used for cell culture were obtained from Sigma.

The cells were cultured on collagen matrices for one week, washed in PBS (phosphate buffer), fixed in 4% paraformaldehyde for one hour, and then cryoprotected. Specimens were frozen in liquid nitrogen and sectioned with a Leica CM 1800 cryotome. The cryosections were washed with PBS and stained with Hoechst 33258 for 15 minutes (a specific DNA staining agent), 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.

Cell viability was determined by MTT assay. The cells cultured on matrices cubes (1mm³) were transferred to a fresh plate and incubated with 0.5mg/mL of MTT during the last 4 h of the culture period tested at 37°C. MTT transformed to dark blue formazan by mitochondrial enzymes

enabling cell viability to be assessed [14]. Then formazan salts were dissolved with 500 μL of lysis buffer (0.1N HCl in anhydrous isopropanol), mixed and the absorbance was measured at 570 nm employing a TECAN 24-well plate reader. The results were expressed as viability percentage.

The cells grown on collagen supports were washed twice with 75mM sodium cacodylate buffer supplemented with 3% (w/v) sucrose (SCB) and fixed in 2.5% glutaraldehyde. Then, the cultures were postfixed in osmium tetroxide for 90 min, at 4°C, washed twice in cacodylate buffer followed by mordanting in 1% (w/v) tannic acid (10 min) and two rinses in 1% (w/v) sodium sulphate [15]. The samples were then rapidly dehydrated in increasing concentrations of ethanol: 70%, 95% and 100% for 5 min followed by Epon 812: ethanol 1:1 (v/v) for 30 min. Finally, the samples were placed in fresh 100 % Epon and polymerized at 55°C for two days. Thin sections obtained on a Reichert OmU3 ultramicrotome were stained with 7.5% (w/v) uranyl acetate and with lead citrate 0.4% (w/v), and examined with a Philips EM 201C and Philips EM 400 (Philips, Holland) transmission electron microscopes. Chemicals used for electron microscopy were obtained from Polysciences (USA).

Results and Discussion

Rheological properties of cross-linked collagen gels depend on the cross-linking agent used. Tannic acid in different concentrations was used as cross-linking agent in order to control the doxycycline release from acid collagen gels.

Collagen hydrogel containing doxycycline was cross-linked with different amounts of tannic acid, and the rheograms are represented in figures 1 (A and B) for 23°C and 37°C (the room temperature and body temperature, respectively).

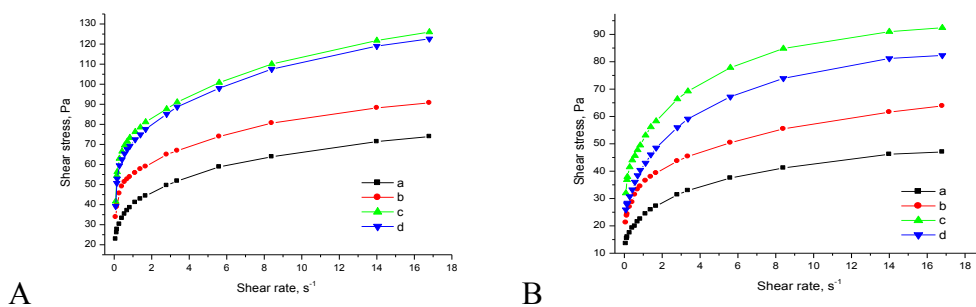


Figure 1

Rheograms of collagen hydrogels cross-linked with:
a) 0; b) 5; c) 10; d) 20% tannic acid at A) 23°C and B) 37°C

The two figures show that all the gels have pseudoplastic behaviour at both temperatures and the shear stress – for all the measured shear rates – increases with increasing the tannic acid concentration until the value of 10% is reached. When tannic acid concentration is 20% the values of shear stresses are lower both at 23°C and at 37°C. This can be due to the unreacted tannic acid, added in excess, which may break the collagen hydrogel structure. Thus, at the maximum shear rate the shear stress for the hydrogel with 10% TA is 1.02 folds higher at 23°C and 1.12 folds higher at 37°C than for the hydrogel with 20% TA. These values show the highest cross-linking degree for hydrogels with 10% TA.

The pseudoplastic behaviour allows hydrogels flowing both at 23°C and 37°C and also casting in trays in order to obtain matrices (spongy form).

Collagen matrices are obtained by the freeze-drying process of collagen solutions or gels. This process consists of two operations: sample freezing in successive stages and sublimation of ice in advanced vacuum. Instead of ice crystals pores are formed, with size varying according to the lyophilizing conditions.

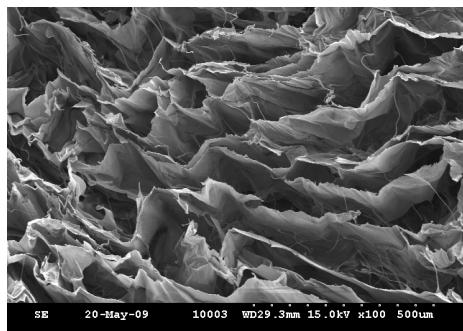
The freeze-drying process results in collagen matrices (porous structures) with pore sizes ranging between 50 and 1500 µm [16] and different shapes, orientation and size.

The effect of collagen cross-linking and of its interaction with the embedded drug on matrix morphology was investigated by pore size measuring using scanning electron microscopy (SEM). Images were captured at different magnification degrees, depending on pore size and how detailed we wanted to get into the matrix structure.

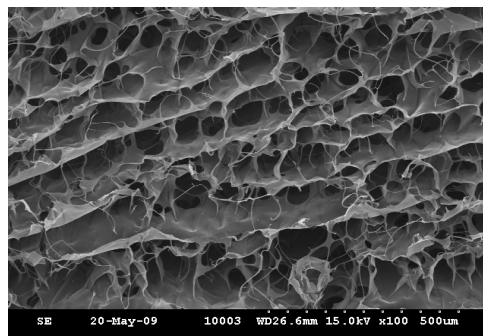
Figure 2 shows the SEM images obtained for M-A – reference collagen matrix at 100x magnification. M-A has staggered lamellar structure with up to 300 µm long and up to 90 µm wide elongated pores interconnected by 0.3 µm diameter collagen fibrils.

Although collagen concentration is the same for all matrices; they have very different structures, when the reference matrix (Figure 2) is compared with those containing doxycycline cross-linked with TA (Figure 3).

The SEM images show that matrices containing TA have more uniform pores and a pore size range between 42 and 312 µm. Their sizes vary between 42 and 260 µm for CD-T5, 33 and 108 µm for CD-T10, and between 86 and 180 µm for CD-T20. CD-T10 matrix, which has the highest cross-linking level, shows the most uniform structure.

**Figure 2**

SEM image of M-A matrix, 100x magnification

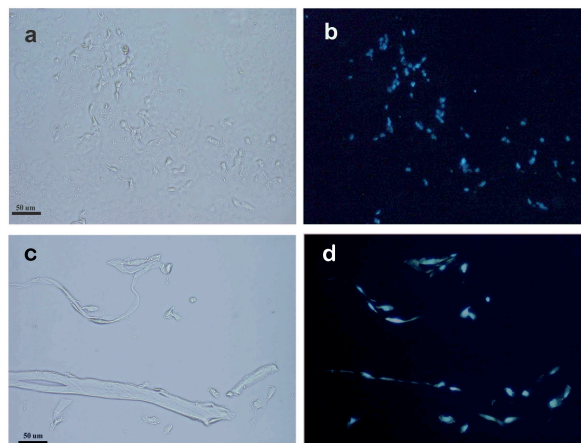
**Figure 3**

CD-T10 matrix SEM image, 100x magnification

The studied collagen matrices pore size varied between 33 and 400 μm , which theoretically allowed the penetration of endothelial cells, the cells having size ranging between 5 and 7 μm .

The *in vitro* evaluation of biocompatibility with human endothelial cells (steady line EA hy 926) shown that both uncross-linked (M-A and CD-A) and cross-linked with TA (CD-T5 and CD-T20) matrices disintegrated in culture medium, therefore cell colonization could not be recorded.

Figure 4 shows images obtained by contrast phase and fluorescence microscopy by nuclear Hoechst staining of endothelial cells colonized on CD-T10 collagen matrices and M (control collagen matrix at 7.3 pH). Three-dimensional collagen matrices allowed the attachment of endothelial cell population and migration due to their porous structure and interconnected pores, forming scaffolds for tissue regeneration.

**Figure 4**

Endothelial cells cultured on collagen matrices: left panels: phase contrast microscopy (a); right panels: blue fluorescent Hoechst staining (b); a, b – CD-T10, c, d – M

The viability of endothelial cells cultured on matrices was 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 (Figure 5).

The ultrastructural examination of the endothelial cells embedded in biocompatible collagen matrices showed the presence of a well developed rough endoplasmatic reticulum, numerous free ribosomes and mitochondria (Figure 6).

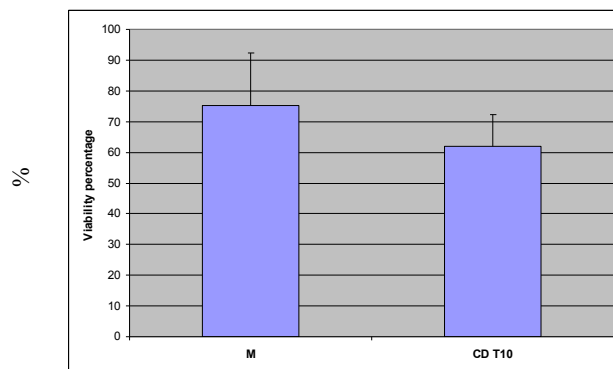


Figure 5

Viability (%) of endothelial cells grown on collagen matrices

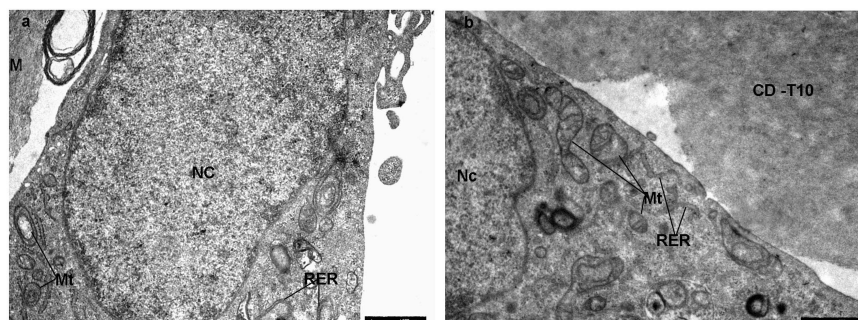


Figure 6

TEM images of endothelial cells grown on collagen matrices: a – M, b - CD-T10

Nc – nucleus, V – vacuoles, RER – rough endoplasmatic reticulum, Mt – mitochondria

Endothelial cells developed normally on collagen matrices revealing that these matrices are biocompatible with this cell type and does not show cytotoxicity, the ultrastructure of endothelial cells remain the same on CD-T10 matrix.

Conclusions

All the studied hydrogels showed a pseudoplastic behaviour. The most viscous was the hydrogel containing 10% TA. Also, the corresponding matrix, CD-T10, presented the smallest pore sizes, between 33 and 108 μm which allow the endothelial cells to penetrate through interconnected pores of matrix. The biocompatibility evaluation showed that all tested matrices were not toxic, but cell colonization could be recorded only on the most cross-linked matrix; the others were disintegrated in culture medium. Endothelial cells presented good viability and ultrastructural examination showed the normal developed cells. The obtained matrices are expected to be used as drug delivery systems for tissue engineering and *in vivo* testing is recommended.

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