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Assessment of cell viability in a three-dimensional enzymatically cross-linked collagen scaffold

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Abstract Microbial transglutaminase (mTGase) is an enzyme that introduces a covalent bond between peptide bound glutamine and lysine residues. Proteins cross-linked in this manner are often more resistant to proteolytic degradation and show increased tensile strength. This study evaluates the effects of mTGase mediated cross-linking of collagen on the cellular morphology, behaviour and viability of murine 3T3 fibroblasts following their seeding into collagen scaffolds. Additionally, cell mediated scaffold contraction, porosity and level of cross-linking of the scaffold has been analysed using image analysis software, scanning electron microscopy (SEM), colorimetric assays, and Fourier transform infrared spectroscopy (FTIR). We demonstrate that the biocompatibility and cellular morphology, when comparing cultures of fibroblasts integrated in mTGase cross-linked collagen scaffolds with the native collagen counterparts, remained unaffected. It has been also elicited that the structural characteristics of collagen have been preserved while introducing enzymatically resistant covalent bonds.

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Introduction

In vitro simulation of in vivo environments is considered as one of the key steps to develop therapeutic interventions in diseased tissues. Collagen is frequently chosen as the primary scaffold for biodegradable implants, as it is the main component of the extracellular matrix mixture of structural and functional proteins [1]. The fact that collagen possesses non allergenic, non-mutagenic, non-migratory properties are contributing factors to its selection for various biomedical applications [1, 2]. Its versatility and ease of manufacture have allowed its fabrication in numerous different forms: ophthalmic shields, sponges, mini-pellets, tablets, gel and nano-particles [1, 3]. However, collagen's limitations in tissue engineering are as well known as its advantages. The rapid in vivo degradation and lack of mechanical strength of the implants has encouraged intensive research in order to overcome these limitations [4].

The aim of this research is the development of a collagen based, collagenase-resistant, enzymatically cross-linked scaffold that maintains the inherent dynamics of the collagen extracellular matrix in relation to cellular viability, proliferation, and migration, as well as tissue reconstruction. Hence the objective of the present study will focus on the evaluation of cellular viability of integrated

fibroblasts in cross-linked and non-cross-linked scaffolds as well as using different physicochemical methods to determine the presence of cross-linking.

Cell viability assays for three-dimensional matrices have not been fully standardised to date. The accessibility of cells to reagents and dyes are markedly different [5, 6] when comparing different scaffolds or different cell locations in the scaffold (on the surface or integrated). Therefore, modifications to the standard methods must be introduced in order to evaluate cell behaviour and viability.

The enzymatic cross-linking agent used to stabilise the scaffold is microbial transglutaminase (mTGase), an enzyme that catalyses a protease-resistant bond between glutamine (Glu) and lysine (Lys) residues [2, 3, 7–10]. The concentration of these amino residues in soluble dermal collagen type I is 27 Glu residues in each α_1 chain and 24 in the α_2 chain [3], and for Lys the concentration is 34 in each α_1 chain and 21 in the α_2 chain. Therefore the concentrations of Glu and Lys in the total amount of amino acids of the atelocollagen α-helix structure are 2.5 and 2.8%, respectively. However these concentrations of aminoacids, when cross-linked, seem to suffice toincrease the scaffold's resistance to proteolytic degradation [4]. We hypothesised that the changes introduced by the creation of the covalent cross-link ε-(γ-glutamyl)lysine by mTGase will not affect cell behaviour and viability but will increase the resistance to contraction. Increased resistance to proteolytic degradation has been shown in previous studies, but unlike this study, three dimensional scaffolds were not used [4].

Materials and methods

All the materials and reagents used in these experiments were supplied by Sigma-Aldrich (Tallaght, Dublin), unless otherwise stated. Abbreviations of the product and catalogue number are specified in parenthesis.

The water used in the different stock solutions was Millipore deionised water (Elix S, Automatic Sanitization Module, AGB Scientific Ltd) and was sterilised in an autoclave at 121 °C for 1 h.

Seeded scaffold preparation

Bovine collagen type I from calf skin (BD Biosciences, Unitech, Dublin, Cat. No. 354231), with a concentration of 2.9 mg/mL and more than 95% purity, was used. Four parts of this collagen solution were gently and thoroughly mixed with one part 5X phosphate buffered saline (PBS, Cat. No: P-4417). The solution was neutralised by the addition of 3 M sodium hydroxide (Cat. No. 30620) until a final pH of 7–7.5 was reached and kept in an ice bath to delay gel formation. The enzyme and/or cells were added at this point depending on the test performed and the group sample.

Microbial transglutaminase (Activa^{TM} WM, Ajinomoto Corporation Inc. Japan) purified by cation-exchange chromatography and whose activity was determined to be 2.7×10^4 nmol of putrescine incorporated/mg/h, was added to the solution at different concentrations (0, 0.025, 0.05 and 0.1 mg/mL).

Subsequently, cells from an immortalised cell-line of 3T3 murine fibroblasts were added to the collagen solution. This suspension was thoroughly mixed, pipetted into the tissue culture plates and kept in a humidified-atmosphere incubator at 5% CO_2 and 37 °C for a minimum of half an hour, until a uniform gelatinous appearance was observed. Dulbecco's Modified Eagle's Medium (DMEM, Cat. No. D6429) supplemented with 1% (v/v) penicillin–streptomycin (Cat. No. P4458), 10% fetal bovine serum (FBS, Cat. No. F7524) and 1% L-glutamine 200 mM (Cat. No. G7513) was carefully added to each well and kept in the incubator until a viability assessment was performed.

In all cell counts, prior to seeding, a standard Trypan Blue (Cat.No:T8158) exclusion assay was performed. Only cell suspensions with viability estimations equal or higher than 99% were used. The cell seeding concentration was maintained constant at 100,000 cells/mL. All the viability assays were performed in attached gels unless stated otherwise.

These fibroblasts integrated in native collagen and cross-linked scaffolds were directly observed after a 24-hour incubation period in an Olympus IX71 microscope (Olympus Corporation, Tokyo, Japan) at ×20 magnification. The direct effect in cellular morphology the level of cross-linking was evaluated.

Viability assays

AlamarBlue^{TM} reduction assay

AlamarBlue^{TM} (BioSource, UK, Cat. No. DAL1025) was used in the evaluation of cellular metabolic activity. The culture media was removed, and the wells rinsed with Hank's Balanced Salt Solution (HBSS, Cat.No: H8264) prior to the addition of 10% (v/v) AlamarBlue^{TM} reagent. The incubation time in all three-dimensional scaffolds was 3 h. This value was found to elicit a high correlation with the data obtained after a standard 1-hour incubation time in a two-dimensional culture. Fluorescence was measured in a FLx800 Microplate Fluorescence Reader (Bio-Tek Instruments, INC.) by exciting at 528 nm and measuring the emission at 590 nm.

The fluorescence readings from the control wells were deducted from the readings obtained from wells containing the scaffolds, and a standard curve was used to extrapolate cell numbers from fluorescence values.

LDH release assay

The integrity of the cell membrane was assessed using CytoTox-ONE™ Homogeneous Membrane Integrity Assay from Promega Corporation (Medical Supplies, Dublin, Cat. No. G7890). The culture media removed in the previous assay (100 μL) was kept at room temperature for 15 min and assessed for LDH release. It was considered that the use of the same culture in the evaluation of two parameters enables a maximum degree of correlation of the results. Fluorescence emission was evaluated using the same filters as per the AlamarBlue™ assay (excitation: 528 nm and emission: 590 nm). A standard curve was created to convert the fluorescent readings obtained into cell numbers and fluorescence values from non-seeded scaffolds were deducted from the values obtained in the seeded ones.

Hoechst 33342 DNA staining

Hoechst 33342 (H342, Cat. No. 33270) assay was used to visually monitor cell density and cell death by apoptosis. The staining pattern indicates the state of the cell: faint and uniform staining of chromatin is visualised in normal cells, increased condensation and brightness is present in early apoptotic cells and chromatin condensation and nuclear fragmentation in late apoptotic cells [11].

From a previously prepared stock solution of 1 mM H342, 100 μL were removed and mixed with 9.9 mL standard cell culture media. After emptying all the wells, 0.5 mL of this dye was added to each well. The plates were kept in a humidified-atmosphere incubator at 5% CO_2 and 37 °C for 90 min to allow penetration of the dye into the cells (minimum required in standard flow-cytometry conditions is 30 min). The fluorescence of the double stranded DNA in the nucleus was detected in ultraviolet light in an Olympus BX51 fluorescent microscope (Olympus Corporation, Tokyo, Japan) at ×40 magnification. All the samples were observed immediately at the end of the incubation period, with a maximum time interval of 2 h between the evaluation of the first and the last sample.

Live and dead assay

Live and Dead® Viability/Cytotoxicity Kit (Invitrogen, Biosciences, Dublin, Cat. No. L-3224) was used to simultaneously visualise live and dead cells. The cytoplasm of the live cells is stained in green (Calcein AM) while the nucleic acids of cells with a compromised cell membrane stain red (Ethidium homodimer-1). In this assay, both probes are simultaneously added to the seeded scaffold at the recommended dilution factor: 10 μL of the supplied 2 mM Ethidium homodimer-1 stock solution and 2.5 μL of the supplied 4 mM calcein AM solution were added to 5 ml of Dulbecco's phosphate buffered saline (D-PBS, Cat No: D8662) tissue culture grade. The solution was vortexed and the wells emptied of culture media prior the addition of 300 μL of the working solution per well. The plate was subsequently incubated (5% CO_2 and 37 °C) for 45 min. The samples were then evaluated with fluorescein long-pass filters sets in an Olympus BX51 fluorescent microscope.

Contraction assays

The cell-seeded scaffolds in 24-well plates were detached from the lateral walls after 24 h of incubation and from the bottom of the wells after 48 h. Culture media was changed every three days or earlier if changes in the colour of the media were detected in the daily observations. The samples were photographed at different time points (5, 7 and 14 days) using GeneSnap (Syngene-Synoptics, Cambridge, UK), an image acquisition software originally developed for electrophoretic gels. The captured images were then evaluated using image analysis software (IMAGE-PRO® PLUS, Media Cybernetics, USA) by calculating the area of the cell-seeded scaffold in relation to the well area. The contraction of the gels is expressed as a percentage of area reduction.

Scanning Electron Microscopy (SEM)

Non-cell-seeded collagen scaffolds were freeze-dried prior to SEM observations to evaluate the porosity of the different scaffolds using area fraction analysis. A VirTis Advantage, Wizard 20 freeze-dryer (SP Industries, New York, USA) was used with the following processing parameters: freeze temperature –25 °C, condenser set-point –45 °C and vacuum set point: 100–200 millitorr.

Additionally cell-seeded collagen scaffolds were visualised on SEM after a four-day incubation period. The samples were fixed with 2.5% glutaraldehyde (GTA, Agar Scientific Ltd., Cambridge, Cat. No: R1011) in 0.1 M sodium cacodylate (Sodium cacodylate trihydrate, Cat. No: C-0250) for 2 h at room temperature, to be subsequently immersed in increasing concentrations of ethanol/water (50, 75, 80, 90 and 100%) for 5 min periods, followed by their immersion in hexamethyldisilazane for 30 min.

All samples, with and without cells, were mounted on carbon pads attached to aluminium stubs and gold coated (Emitech K550 Sputter Coater, Emitech Limited, Ashford, Kent, UK). A Hitachi Scanning Electron Microscope S-4700 (Hitachi-Hisco Europe GmbH, Berkshire, UK) was utilised in the visualisation of the samples.

The porosity of the scaffold was evaluated using the area fraction methodology, where a minimum of three SEM images (×250 magnification) of each scaffold was evaluated. A fixed size grid was superimposed on all images and the intersections were tagged with matrix or pore accordingly. The pore area fraction was calculated and plotted as number of pore-tagged intersections by the total number of intersections in the image.

Evaluation of cross-linking

TNBS assay

The level of cross-linking in the scaffolds was evaluated by the quantification of free amino groups using 2,4,6-Trinitrobenzene Sulfonic Acid 5% (w/v) (TNBS, Pierce, Medical Supplies, Dublin, Cat. No: 28997) by a method developed by Bubnis et al. [12, 13]. Freeze-dried cross-linked and non-cross-linked collagen samples were weighed (2–4 mg) and placed in glass tubes. One ml of sodium bicarbonate and 1 mL of 0.5% (v/v) TNBS were added to each tube prior to a 3-hour incubation at 40 °C. After cooling and adding 3 mL of 6 N hydrochloric acid (HCl 37%, Cat No: 30721) to each tube, the samples were kept in the oven for another 2 h at 60 °C. The solution was allowed to cool and diluted with 5 mL of deionised water. Absorbance was measured in the spectrophotometer (UV-visible Spectrophotometer, UV-1601, Shimadzu Biotech Corporation, Germany) at 346 nm against previously prepared blanks.

The cross-linking values were expressed in moles of Lysine per moles of collagen using the following formula:

$$\mathrm{ML/MP} = 2(\mathrm{ABS})\ (\mathrm{DF})\ (\mathrm{MW}) \Big/ [(1.46 \times 10^4 \mathrm{liters/mole.cm})(\mathrm{b})(\mathrm{x})]^{-1}$$

where ML/MP: Moles of ε-amino groups/moles of protein, ABS: absorbance, DF: dilution factor, 0.05, MW: molecular weight of the protein, b: the cell path length in cm, x: sample weight in grams.

Infrared spectroscopy

Fourier transform infrared (FTIR) spectroscopy was used to determine the presence of cross-linking and structural changes in our scaffolds. Infrared (IR) spectra were recorded from films with a Shimazu FTIR-8600 (Shimazu, Japan) at room temperature, with an interval of 2 h between the first and the last measurement. An average of 40 scans was obtained in each spectrum.

In the preparation of the films, 100 μL of neutralised cross-linked and non-cross-linked collagen gel was placed on tissue culture coverslips of 13 mm diameter (Sarstedt, Newton, USA, Cat. No. 83.1840.002) and incubated (5% CO_2 and 37 °C) overnight. The films were dried for 4 h at room temperature in a laminar flow fume-hood before measuring the infrared spectra. The readings of empty coverslips were labelled as background measurements prior to spectra readings.

Statistical analysis

One way analysis of variance (ANOVA) was used to evaluate the data with *post*-hoc differences between groups identified using Bonferroni *t*-test (MINITAB™ version 13.32, Minitab, Inc.). A *p*-value less than 0.05 was considered to be statistically significant.

Results

Cell morphology

The morphological appearance of cells cultured in native collagen does not markedly differ from the ones cultured in the cross-linked counterparts, as observed in Fig 1. The low cell seeding density and short incubation period allow the visualisation of the cells and their processes without image overlap. Dendritic phenotype is elicited by most of the seeded fibroblasts for all mTGase concentrations. The main body of the cells is round or elongated projecting this dendritic-like network of extensions in all directions interconnecting with neighbouring cells. Blurred profiles of cells located at different focal distances can additionally be evidenced. This phenotype normally corresponds to cells seeded in free floating gels leading us to believe that the mechanical loading of the surrounding matrix is low and does not increase with the level of cross-linking.

Viability assays

AlamarBlue™ reduction assay

The cytotoxicity of mTGase was assessed by evaluating the effect that different concentrations of the enzyme (0, 0.025, 0.05 and 0.1 mg/mL) have on the metabolic activity of 3T3 fibroblasts at different time points: 24 h, 48 h and 5 days.

Groups of different concentrations of mTGase were statistically compared at different time points (Fig. 2). There was no significant difference ($p \leq 0.05$, expressed in the figure with * when present) in the cellular metabolic rate between scaffolds for all mTGase concentrations used.

LDH release assay

The LDH release evaluated in the culture media indicated no statistical increase in the LDH leakage when comparing

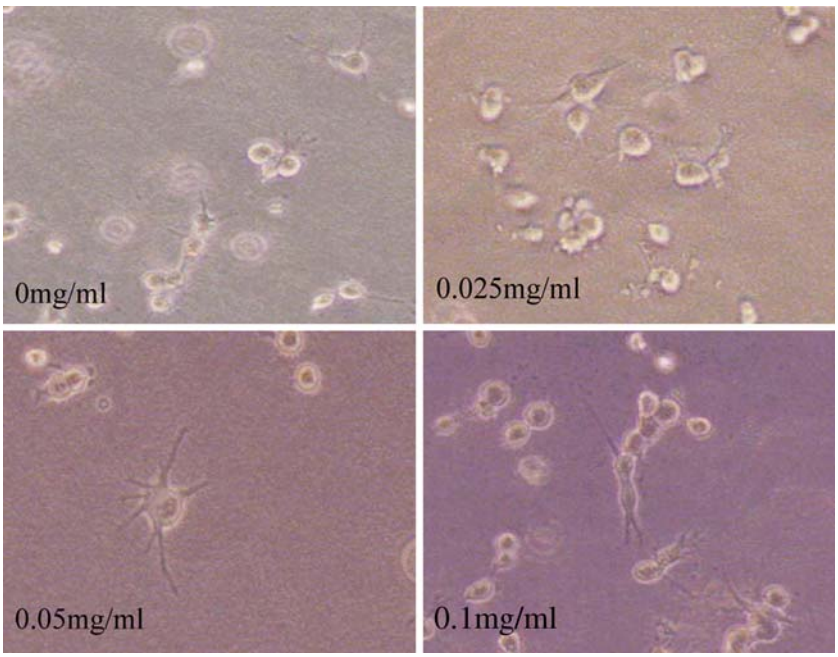

Fig. 1 Bright field images of scaffolds with different concentrations of mTGase at ×20 magnification

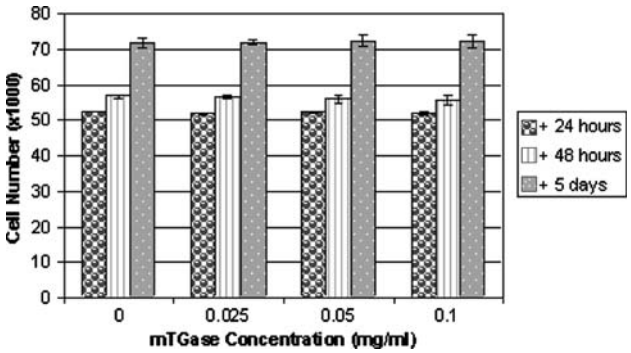

Fig. 2 AlamarBlue™ reduction assay in three dimensional collagen scaffolds seeded with 100,000cells/ml and cross-linked with three different concentrations of mTGase (n = 3)

the non-cross-linked and the cross-linked collagen scaffolds seeded with the same cell density and under the same environmental conditions (Fig. 3).

Hoechst 33342 DNA staining

The size, density and morphological appearance of the nuclei elicited by the cells immersed in a non-cross-linked collagen scaffold (Fig. 4) did not differ from the cross-linked scaffolds, after a 48-hour incubation period. A minimum of five focal fields were evaluated in all cases, n = 3. Isolated evidences of apoptosis were observed in a few samples where the nucleus seemed to have an irregular shape. However, no more than one of these suspected early apoptotic cells was observed per field in any given sample, and no correlation was established between these findings and cross-linked scaffolds, leading us to believe that mTGase does not induce cellular apoptosis.

Live and dead assay

The Live and Dead assay evidenced no cytotoxic effects on the seeded cross-linked scaffolds, as observed in Fig. 5. Fluorescence background is emitted by the cells that are at different focal distances, as should be expected in a three dimensional seeded matrix. Higher cell densities of cells were observed in the periphery of the well due to the menisci effect present in attached scaffolds.

Contraction assays

The effect that different concentrations of mTGase had on preventing cell-mediated contraction in the collagen scaf-

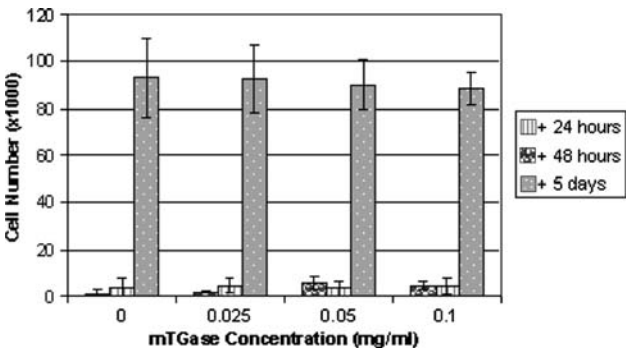

Fig. 3 LDH release assay in three-dimensional collagen scaffold seeded with 100,000 cells/mL and cross-linked with three different concentrations of mTGase (n = 3)

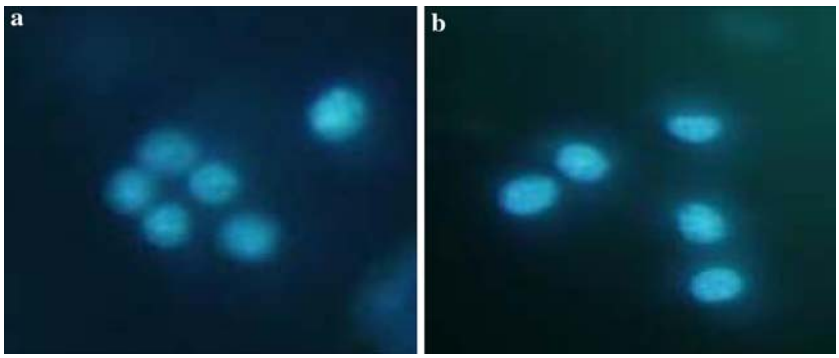

Fig. 4 (**a**) Shows the nuclei of cells seeded (100,000cell/mL) in a non-cross-linked collagen scaffold after 48 h incubation and (**b**) shows the nuclei of the cells of the cross-linked (mTGase concentration of 0.5 mg/mL) counterpart

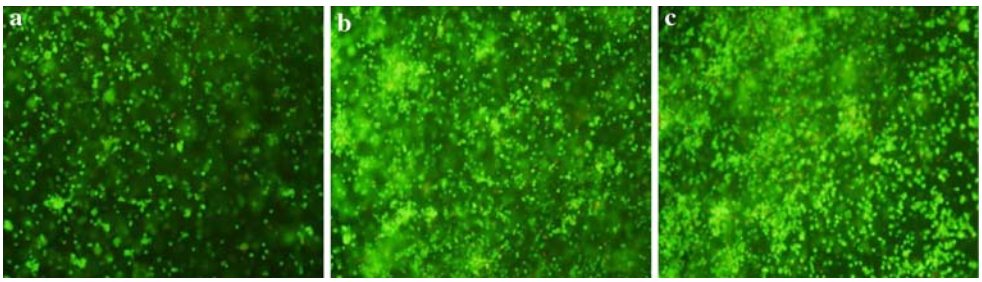

Fig. 5 Photographs of Calcein AM and Ethidium homodimer-1 stained cells in three-dimensional scaffolds after 5 days of incubation and an original cell density of 100,000 cell/mL. (**a**) control collagen scaffold (0 mg/mL mTGase) (**b**) cross-linked collagen scaffold with 0.025 mg/mL of mTGase and (**c**) collagen cross-linked scaffold with 0.1 mg/mL of mTGase (n = 3)

folds was evaluated at days 5, 7 and 14. After 14 days and prior to 5 days, no significant changes were perceived in scaffold size with respect to the established time points ($p > 0.05$). The results, as in Fig. 6, indicate that mTGase does not prevent cell-mediated contraction of the cross-linked scaffolds when compared with the control at any given time point.

Scanning Electron Microscopy (SEM)

Area fraction of pores

Visual changes in the scaffold's structure or the distribution of the different interconnecting pores cannot be easily elucidated when comparing the SEM images of control with mTGase cross-linked scaffolds, as evident in Fig. 7. A more objective evaluation of the porosity was achieved by using area fraction analysis. The porosity of non cross-linked scaffolds was significantly smaller when compared with any of the cross-linked scaffolds. These results infer that the scaffold porosity increases with the degree of cross-linking (Fig. 8).

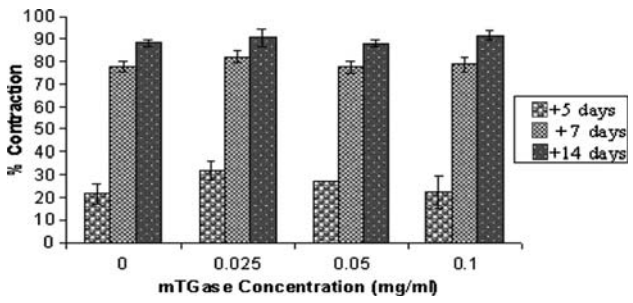

Fig. 6 Contraction rate of cross-linked and non-cross-linked scaffolds (expressed in percentage) after 5, 7 and 14 days of incubation time (n = 3)

Cell seeded scaffold

The SEM images of seeded collagen scaffolds in Fig. 9 show cells immersed within the collagen fibre network, emitting cytoplasmic expansions to link with each other or with the matrix creating an incipient tissue-like structure. No obvious morphological differences are detected when using 0, 0.025 and 0.5 mg/mL of mTGase. However 0.05 0.1 mg/mL mTGase cross-linked scaffold seems to resist the mechanical force exerted by the fibroblasts giving the cells a stellate/bipolar shape analogous to cells cultured on more rigid surfaces.

Evaluation cross-linking

TNBS assay

The extent of cross-linking with different concentrations of mTGase against a positive control of 2.5% of glutaraldehyde was measured and the obtained values were plotted

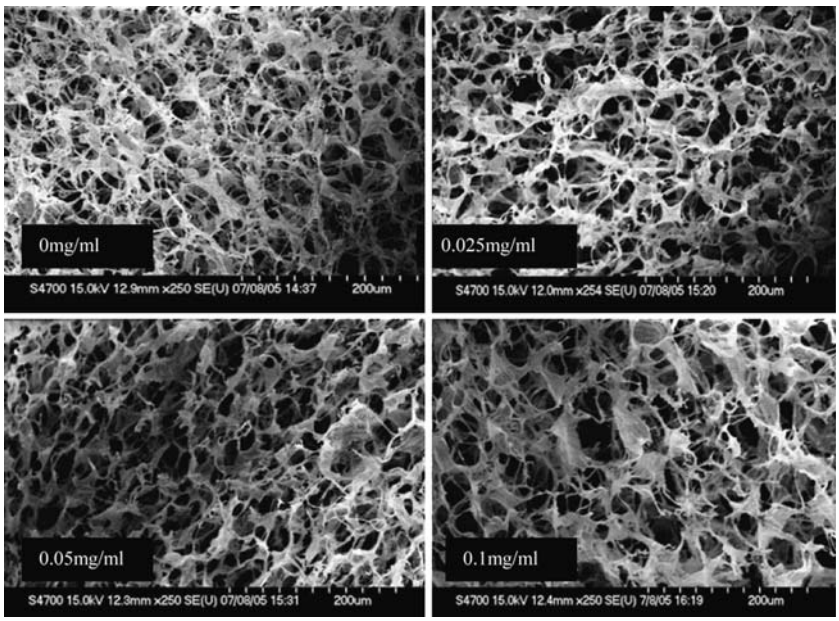

Fig. 7 SEM images of scaffolds with different concentrations of mTGase at ×250 magnification

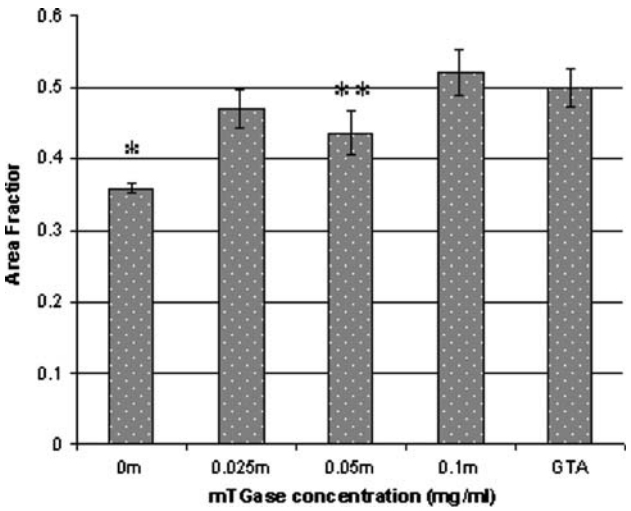

Fig. 8 Pore area fraction in different scaffolds with different concentrations of mTGase ($n = 3$). (*): There is statistical difference between control and cross-linked scaffolds, $p < 0.05$. (**): Means statistical difference between 0.05 mg/mL mTGase cross-linked scaffold and 0.1 mg/mL mTGase and glutaraldehyde scaffolds, $p < 0.05$

on a graph (Fig. 10). This graph indicates that increasing concentrations of mTGase resulted in a decrease in the amount of free lysine residues. The glutaraldehyde cross-linked positive control was statistically different to all other groups, $p < 0.05$. The first three groups (0, 0.025 and 0.05 mg/mL mTGase concentration) do not show any statistical difference in the degree of cross-linking among themselves. On the other hand 0.1 mg/mL mTGase cross-linked scaffold shows marked difference when compared with the other groups inferring that the amount of free amino groups is reduced in the same proportion.

FTIR analysis

The infrared spectra for the collagen scaffold with varying concentrations of mTGase are shown in Fig. 11. Pure collagen spectra have 4 characteristic bands: Amide A, B, I and II. The bands for collagen normally appear at around 3330, 3080, 1650–1690 and 1550 cm^{-1} [14, 15]. In our spectra, (Table 1) amide A is found at around 3310–3330 cm^{-1}, amide I band at 1710 cm^{-1} and amide II at around 1650 cm^{-1} with no changes in amide B. The intensity of the bands varies with the degree of cross-linking. With increasing concentrations of mTGase the intensity of the amide A band decreases whereas that of amide I increases, see in Fig. 11. Additionally the absorbance ratio from bands at 1235 and 1450 cm^{-1} was calculated to verify the integrity of the triple helix structure [16]. All values obtained are greater than 1 as elicited in Table 1.

Discussion

The viability of 3T3 murine fibroblasts integrated in a three-dimensional enzymatically cross-linked collagen scaffold was evaluated according to different parameters:

1. Metabolic activity, by measuring the reducing environment of the cell.
2. Cell membrane integrity, by measuring the leakage of LDH in the media and dye permeation to ethidium homodimer-1.
3. Cell death by apoptosis, by DNA staining.
4. Esterase activity of live cells, by calcein AM retention.

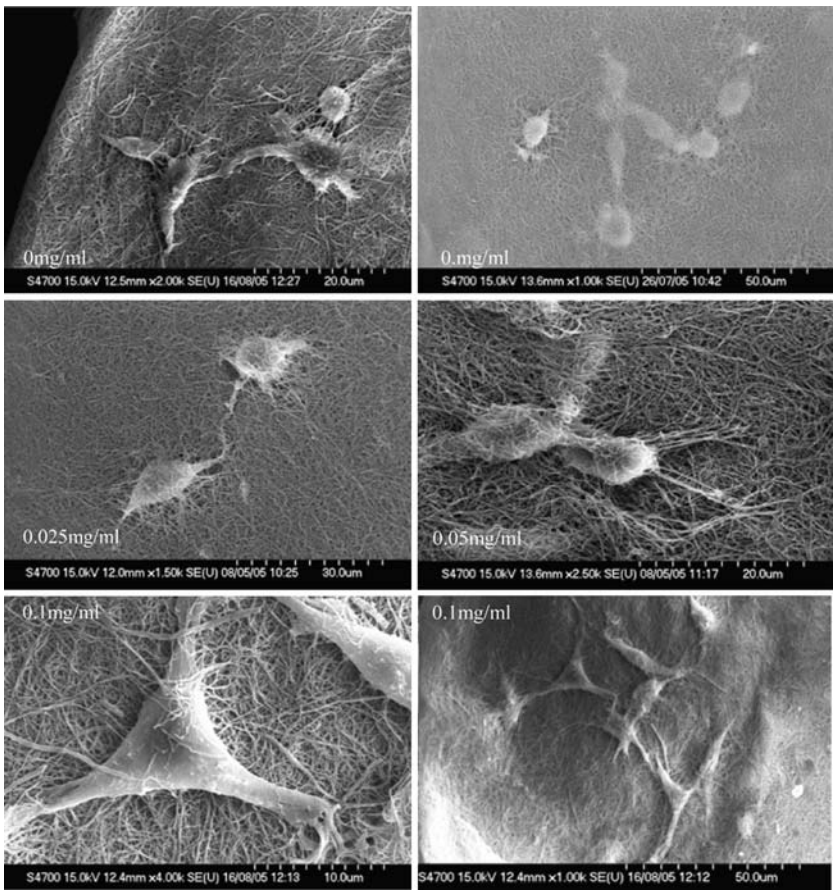

Fig. 9 SEM images of cells within collagen cross-linked and non-cross-linked scaffolds, the mTGase concentrations used in each case are shown on the images

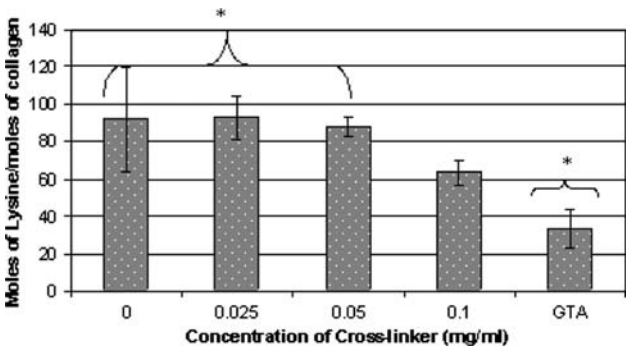

Fig. 10 Moles of free lysine residues in relation to different levels of cross-linking (n = 3). (*): Denotes statistical difference between the first three groups when compared with glutaraldehyde cross-linked scaffold

Standardisation of cell viability and proliferation assays in three dimensional matrices has been previously attempted [17] and the derived conclusions are only limited to the particular specifications of a given experiment. As a consequence, modifications to the original protocols were performed to adapt them to our working conditions.

Fibroblasts integrated in collagen scaffolds express tissue-like phenotype not observed in monolayer cultures or plastic [5]. Knowing the state of mechanical loading of the cultured fibroblasts is essential to interpret the biosynthetic activity [18–20], the capacity of extracellular matrix (ECM) remodeling [21, 22], the proliferation rates [20, 23], and the morphological appearance [5] exhibited by the seeded fibroblasts. The proliferation rate of fibroblasts immersed in collagen gels lowers when compared with monolayer conditions [5] and a small percentage of fibroblasts become quiescent or apoptotic within 24 h of absence of mechanical load [6]. Additionally collagen synthesis increases and collagenase activity decreases in cross-linked scaffolds [22], which has a direct effect in ECM remodeling [21, 24, 25]. This is due to the correlation cross-linking to mechanical stiffness of the substrate [20]. With regard to the cellular morphology, fibroblasts cultured in collagen scaffolds develop more connective tissue appearance in contrast to the flattened, lamellar appearance developed in planar surfaces [26, 27]. The effect of mechanical loading in cell morphology is even more dramatic when stress-relaxation stimulation is performed. In this case fibroblasts cultured in gels that remain attached to the walls have stellate/bipolar shape. However if the edges of the gel are released, the cells become rounded with retraction of the pseudopodia and the actin bundles col-

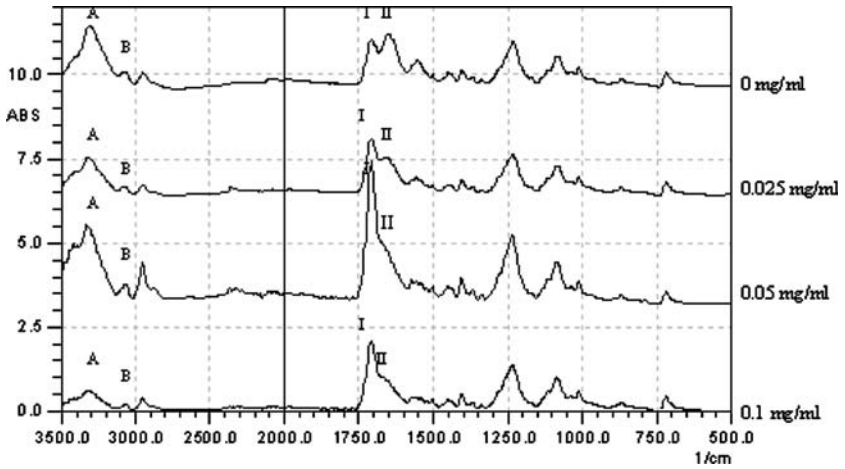

Fig. 11 FTIR spectra obtained from films treated with different concentrations of mTGase (0, 0.025, 0.05, 0.1 mg/mL)

Table 1 Standard infrared wavelengths for the different bands are indicated on the left of the table

Bands	Standard WL (cm^{-1})	0 mg/mL WL (cm^{-1}) ABS		0.025 mg/mL WL(cm^{-1}) ABS		0.05 mg/mL WL(cm^{-1}) ABS		0.1 mg/mL WL (cm^{-1}) ABS	
Amide A	3330	3313.5	1.6	3315.4	1.1	3328.9	1	3311.5	0.6
Amide B	3080	3080	0.9	3080	0.3	3080	0.26	3080	0.2
Amide I	1655	1708/	1.6	1708.8	1.7	1710.7	1.87	1710.7	2.1
Amide II	1550	1647.1	1.8	1662	1.2	1655	0.7	1655	0.9
Amide III	1250	1234.4	2	1234.4	1.3	1236.3	0.87	1236.3	1.4
Collagen Degradation	1235/1450	3.7 (0 mg/mL)		3.5 (0.025 mg/mL)		4 (0.050 mg/mL)		4.2 (0.1 mg/mL)	

Measured infrared wavelengths (WL) for the samples are to the right hand side with the correspondent absorbances (ABS)

Additionally the ratio of the absorbances at 1235 and 1450 cm^{-1} are expressed for the different cross-linking conditions ($n = 40$)

lapse [28]. Therefore, it is paramount for the interpretation of the results to state that the cells were seeded within the scaffold and that the scaffolds were not detached from the walls to keep them mechanically loaded.

The common denominator of the viability assays used in this research is that the assays allow the assessment of the cell viability with minimal disturbance to the in vitro environment. This approach ensures an accurate appraisal of the cellular environmental conditions provided by the scaffolds and cellular response to them. Other important considerations when choosing viability assays were the possibility of performing more than one assay per well allowing maximal correlation of results; maximal diversity of the parameters measured and the feasibility of continuous monitoring of the cell cultures, where continuous evaluation of cell behaviour could be made over time.

AlamarBlue™ reduction assay allows continuous monitoring of the cells in culture without interfering with proliferation or viability [29]. This assay shows an increase in cell numbers after 24 h of culture with no significant difference between different cross-linker concentrations. Corroborating similar observations reported by Chevallay et al. where the proliferative and biosynthetic behaviour of fibroblast in cross-linked scaffolds was similar to the behaviour exhibited in attached collagen gels [22]. However Ng et al. detected a lack of linear correlation in the AlamarBlue™ reduction assay when working with high cell densities. This preceding research that we have seen could explain the result observed on day 5 after seeding [17]. The marked increase in LDH release on day five does not correspond with a similar increase in cell number. We hypothesise that at the five-day time point, cells could have gone into arrest, reaching a certain degree of confluency where fibroblasts ceased being under mechanical stress and have become apoptotic [6], resulting in an increase of LDH release.

On a more qualitative basis, the visual interpretation of early apoptotic figures when using DNA staining is more challenging when using 3T3 murine fibroblasts. The nuclei of these cells have condensations of chromatin uniformly distributed in normal physiological conditions. Therefore only cells, which showed small nuclear size with increased chromatin density, irregular distribution of these condensations and/or irregular nuclear outline were regarded as apoptotic [11].

Fluorometric evaluation of cell viability with Live and Dead Assay was attempted with a microplate reader in order to provide a more quantitative tool. Inconclusive

results were obtained as the three-dimensional nature of the seeding induced overlapping of the fluorescence emitted by the stained cells.

Cells fixed in cross-linked scaffolds were observed by electron scanning microscopy. The scaffold corresponding to 0.05 mg/mL of mTGase shows evidence of collagen fibrils being pulled by the cells as well as cellular protrusions establishing intracellular contacts. In 0.025 mg/mL group the outline of two interconnecting cells is detected on a deeper plane, as they are integrated in the matrix. Evidence of cells emerging to the surface of the scaffolds is seen on the images taken at lower magnifications (1.00 K) where silhouettes/protrusions of cells can be distinguished (Fig. 9). The characteristic stellate shape of fibroblasts growing on isotropic substrates is discerned at the maximum concentration of mTGase used (4 K). The observed morphology can be explained by the fact that the fibroblasts emerge and proliferate on the surface. Therefore these cells will exhibit a mixed phenotype between monolayer and three-dimensional cultures [5] hence it can be inferred that these fibroblasts have modified their phenotype according to the location in the scaffold.

The evaluation of the different behavioural parameters of proliferation and matrix contraction together with the evidence of fibroblast-like phenotypic features allow us to conclude that mTGase cross-linked scaffolds not only are not toxic but they provide ideal conditions of cell growth and differentiation.

When evaluating scaffold cross-linking, TNBS assay showed that only when using concentrations of mTGase of 0.1 mg/mL was possible to obtain levels of cross-linking closer to the glutaraldehyde's. This finding is extremely promising for future work in dermal tissue engineering, as we have no evidence of toxic effects at that concentration. FTIR spectroscopy further proved minimal structural changes with the introduction of covalent links. Amide II band originates on the bending of N–H bonds [28] and is characteristic of triple helical proteins [17]. This band is present in all samples, despite the fact that its absorbance decreases with the increase in cross-linking. However, it is amide A band, originating on the asymmetric stretch of NH bands and characteristic of NH_2 groups, the band that suffers a more drastic decrease in its absorbance as the cross-linking takes place. In the formation of the covalent bond the γ-carboxamate group (–C = O) of the peptide bound glutamine, transfers an acyl group to the ε-amino group ($-NH_2$) of peptide-bound lysine, resulting in the formation of ε (γ-glutamyl)lysine iso-peptide bond and liberation of ammonia [7, 8, 30, 31]. Therefore as the cross-linking takes place there are less NH_2 groups free, as evidence by the fact that amide A is decreasing. The intensity of $-NH_2$ band in collagen molecules is stronger than that of NH [16]. Conversely amide I absorption band increases with the level of cross-linking correlating with an increase in stretching of C = O and bending of N–H in the new covalent bonds between glutamine and lysine [16, 29].

Pores of the cross-linked scaffolds are bigger than their equivalents in the native scaffold due to the tighter assembly of the collagen fibrils. Finding that supports that the enzymatic resistance could be related with tightening in the collagen's conformation of the triple helix structure at and near the cleavage site (775–776 amino residues). Therefore recognition and binding of collagenase to the relevant residues is avoided and/or delayed [32].

On the other hand these new bonds introduced by mTGase seem insufficient to significantly modify the mechanical characteristics of this cross-linked scaffold as seen in the cell mediated contraction. This finding potentially favours cell migration and proliferation within the scaffold at the same time that preserves the collagen's versatility. However if the introduction of covalent bonds reduces the release of cellular metalloproteinases by increasing the mechanical load of the scaffold, we could infer that the same event will take place in our working conditions. This will contribute to the scaffolds resistance to protein degradation with the subsequent positive consequences in tissue formation and remodelling.

In conclusion, as inferred from the results, mTGase cross-linked scaffold supports cell viability while cell mediated contraction activity remains unaffected. Therefore, the use of mTGase to enzymatically stabilise collagen for potential use as a scaffold in tissue engineering applications is extremely promising and broad.

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References

1. C. H. LEE, A. SINGLA and Y. LEE, *Int. J. Pharm.* **221** (2001) 1
2. M. D. FERNANDEZ-DIAZ, P. MONTERO and M. C. GOMEZ-GUILLEN, *Food Chem.* **74** (2001) 161
3. W. FRIESS, *Eur. J. Pharm. Biopharm.* **45** (1998) 113
4. D. Y. S. CHAU, R. J. COLLIGHAN, E. A. M. VERDERIO, V. L. ADDY and M. GRIFFIN, *Biomaterials* **26** (2005) 6518
5. B. ECKES, P. ZIGRINO, D. KESSLER, O. HOLTKOTTER, P. SHEPHARD, C. MAUCH and T. KRIEG, *Matrix. Biol.* **19** (2000) 325
6. F. GRINNELL, *Trends Cell Biol.* **10** (2000) 362
7. M. GRIFFIN, R. CASADIO and C. M. BERGAMINI, *Biochem. J.* **368** (2002) 377
8. R. SHARMA, P. C. LORENZEN and K. B. QVIST, *Int. Dairy J.* **11** (2001) 785

9. M. MOTOKI and K. SEGURO, *Trends Food Sci. Technol.* **9** (1998) 204
10. P. GRENARD, S. BRESSON-HADNI, S. EL ALAOUI, M. CHEVALLIER, D. A. VUITTON and S. RICARD-BLUM, *J. Hepatol.* **35** (2001) 367
11. S. ALLEN, J. SOTOS, M. J. SYLTE and C. J. CZUPRYNSKI, *Clin. Diagn. Lab. Immunol.* **8** (2001) 460
12. W. A. BUBNIS and C. M. OFNER 3rd, *Anal. Biochem.* **207** (1992) 129
13. C. M. OFNER 3rd and W. A. BUBNIS, *Pharm. Res.* **13** (1996) 1821
14. A. KAMINSKA and A. SIONKOWSKA, *Polym. Degrad. Stab.* **51** (1996) 15
15. X. H. WANG, D. P. LI, W. J. WANG, Q. L. FENG, F. Z. CUI, Y. X. XU, X. H. SONG and M. VAN DER WERF, *Biomaterials* **24** (2003) 3213
16. D. GOPINATH, M. R. AHMED, K. GOMATHI, K. CHITRA, P. K. SEHGAL and R. JAYAKUMAR, *Biomaterials* **25** (2004) 1911
17. K. W. NG, D. T. LEONG and D. W. HUTMACHER, *Tissue Eng.* **11** (2005) 182
18. P. FRIEDL and E.-B. BRÖCKER, *Cell Mol. Life Sci.* **57** (2000) 41
19. C. A. LAMBERT, A. C. COLIGE, C. MUNAUT, C. M. LAPIERE and B. V. NUSGENS, *Matrix Biol.* **20** (2001) 397
20. F. GRINNELL, *Trends Cell Biol.* **13** (2003) 264
21. M. CHIQUET, A. S. RENEDO, F. HUBER and M. FLUCK, *Matrix Biol.* **22** (2003) 73
22. B. CHEVALLAY, N. ABDUL-MALAK and D. HERBAGE, *J. Biomed. Mater. Res.* **49** (2000) 448
23. T. L. LEE, Y. C. LIN, K. MOCHITATE and F. GRINNELL, *J. Cell Sci.* **105**(Pt 1) (1993) 167
24. R. T. PRAJAPATI, B. CHAVALLY-MIS, D. HERBAGE, M. EASTWOOD and R. A. BROWN, *Wound Repair Regen.* **8** (2000) 226
25. M. CHIQUET, *Matrix Biol.* **18** (1999) 417
26. F. GRINNELL, C. H. HO, E. TAMARIZ, D. J. LEE and G. SKUTA, *Mol. Biol. Cell* **14** (2003) 384
27. E. TAMARIZ and F. GRINNELL, *Mol. Biol. Cell* **13** (2002) 3915
28. M. EASTWOOD, V. C. MUDERA, D. A. MCGROUTHER, R. A. BROWN, *Cell Motil. Cytoskeleton* **40** (1998) 13
29. M. M. NOCIARI, A. SHALEV, P. BENIAS and C. RUSSO, *J. Immunol. Methods* **213** (1998) 157
30. L. LORAND and R. M. GRAHAM, *Nat. Rev. Mol. Cell Biol.* **4** (2003) 140
31. K. YOKOYAMA, N. NIO and Y. KIKUCHI, *Appl. Microbiol. Biotechnol.* **64** (2004) 447
32. B. BRODSKY and J. A. M. RAMSHAW, *Matrix Biol.* **15** (1997) 545