

**Thesis for the Degree
of
Master of Science**

**Development of Porous Gelatin Scaffolds
for Animal Cell Culture**

**by
Yarasca De La Vega Karen**

**Major in Molecular Medical Science
Department of Life Science
School of Life Sciences and Biotechnology**

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Development of Porous Gelatin Scaffolds for Animal Cell Culture

By Yarasca De La Vega Karen

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Written under the direction of Professor Ik-Hwan Kim

Dissertation Committee:

Professor Ik-Hwan Kim _____

Professor Man-Bock Gu _____

Professor Chan-Wha Kim _____

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List of Abbreviations

FBS	Fetal Bovine Serum
MEM-α	Minimum Essential Medium-Alpha
DMEM	Dulbeccos's modified eagle's medium
PBS	Phosphate Buffered Saline
SEM	Scanning Electron Microscopy
GTA	Glutaraldehyde
DL-Gly	DL-Glyceraldehyde
DMS	Dimethyl suberimidate dihydrochloride
EDC	N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
CCK-8	Cell Counting Kit-8
Vero	Monkey Kidney Fibroblast Cell Line
HAC	Human Articular Chondrocyte

Abstract

The physical and biological properties of gelatin-based biomaterials are profoundly influenced by the method of crosslinking. The aim of this study was to investigate the influence of various crosslinking treatments for generating stable porous gelatin scaffolds to optimized cell culture lines. Several different methods were used to cross-link the porous gelatin scaffolds, including glutaraldehyde (GTA), DL-glyceraldehyde (DL-Gly), and Dimethyl suberimidate dihydrochloride (DMS), as well as N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC). The swelling ratio, the morphology of the scaffolds, resistance to collagenase and PBS digestion, and *in vitro* cell culture were performed to evaluate the physical and biological properties of crosslinked matrices. Because GTA has been shown to be cytotoxic, other cross-linking agents were explored as an alternative to avoid those side effects. However, all the crosslinked scaffolds were degraded after only few hours in aqueous medium containing collagenase at 37°C.

Moreover, Vero and HAC cell lines were grown on each of the cross-linked scaffolds and a proliferation assay showed that the cells concentration and cell viability is as well or better on the scaffolds crosslinked with GTA and DL-Gly than on traditional monolayer polystyrene culture plates. These results have shown promising potential of using gelatin-based matrices crosslinked with DL-Gly because of the low toxicity, good cell proliferation and cell viability of this reagent and the resistance to dissolution of the cross-linked scaffold in cell culture medium for over 2 weeks at 37°C.

Introduction

Gelatin is the product of thermal denaturation or disintegration of insoluble collagen [1], and since it retains some of its precursor chemotactic signals (e.g., RGD amino acid sequence, which promotes cell adhesion [2] and migration [3, 4, 5]), it has been considered as a cost-effective alternative to collagen for the potential application in tissue engineering [6, 7]. Also, the non-antigenicity of gelatin has attracted attention by Starin [8], in particular, who carried out an extensive investigation, using the precipitin, anaphylactic, complement fixation and meiotagmin reactions, and decided that the injection of gelatin into rabbits, guinea-pigs and dogs failed to produce antibodies by gelatin [9].

In the last years, gelatin-based biomaterials have been applied to artificial skin, bone grafts, and scaffolds for tissue engineering [10-13]. In tissue engineering, scaffolds provide a platform for cell attachment, migration and differentiation [14]. For the scaffold to be able to function optimally in this role, porosity within the structure is a key element so that efficient nutrient transmission may take place [15].

For many years the freeze-drying technique has been widely adopted in pharmaceutical [16, 17] and food industries [18, 19], since this technique provides increased stability or rapid solubility in many applications, such as drug delivery including site-specific and antibiotic delivery systems. In this study, I applied a method using only water as a porogen and the freeze-drying technique to produce highly porous scaffolds from biodegradable gelatin. Because after freezing the gelatin solutions or the swollen scaffolds, the ice formed within the hydrogel network was sublimated by freeze-drying. This simple method produced porous hydrogels, without use of any additives and organic solvents [20].

Nevertheless, the main limitation of gelatin for application in tissue engineering is that it is soluble in aqueous environments. The method to relieve this limitation is using crosslinking agents [21, 22]. However, the toxicity of the crosslinking agent should be taken into serious consideration in developing biomaterials.

The objective of the present study is to assess the effect of various crosslinking methods on the physical and biological properties of gelatin scaffolds. The crosslinking agents used included glutaraldehyde (GTA),

DL-glyceraldehyde (DL-Gly), diimidoesters-dimethyl suberimidate (DMS) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) at their respective optimal conditions. The influence of crosslinking on the swelling behavior, in the biodegradation and in kinetics cell culture properties of the gelatin scaffold samples were assessed.

GTA was used to stabilize the scaffolds and to tailor their mechanical properties. GTA is a very effective crosslinking agent which functions by establishing covalent bonds with the amine groups of lysine [23]. These reactions increase the mechanical strength and thermal stability [24], as well as life span [25] of the scaffolds. However, glutaraldehyde in certain concentrations has been shown to be cytotoxic [26, 27]. For that reason new methods were selected for this study based on the premise of reduced toxicity relative to glutaraldehyde.

To overcome the restriction of using crosslinked gelatin in the pharmaceutical field, DL-Gly, a non-toxic crosslinking agent, was proposed [28, 29]. DL-Gly has been used to cross-link gelatin and is nontoxic. Furthermore, glyceraldehyde is a natural product of a metabolic

process [29-31]. Studies suggest that gelatin crosslinked with glyceraldehyde is well tolerated in vivo. Moreover, the D form of this aldehyde is phosphorylated by triosekinase in the human body forming D-glyceraldehyde- 3-phosphate that enters in glycolysis cycle [29].

Several studies have demonstrated that collagen membranes crosslinked with DMS are shown to be more biocompatible than those treated with GTA [9, 32]. Also, it is known that DMS do not polymerize and form crosslinks only with free amino groups [23]. Thus, these properties could be applied also to crosslink gelatin scaffolds, working with the optimal concentration and conditions used in collagen membranes.

EDC was recently known to be non-toxic and bio-compatible agent due to easy removal of excess reagent by washing with dilute acid or water. Because it is not incorporated into the crosslinked structure, but simply changed to a water-soluble urea derivative [26, 33, 34].

Several assays were performed to analyze the influence of these crosslinking agents over the performance of the porous gelatin scaffold when it is used to culture different cell lines.

Materials and Methods

1. Materials

1.1. Chemical and reagents

Type B gelatin powder from bovine skin and Collagenase from *Clostridium histolyticum* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized sterile distilled water were obtained from BIONEER Corp. (Daejeon, South Korea). Enhanced cell viability assay kit CCK-8 (EZ-cytox, DogenBio Co, Korea) was purchased to cytotoxic analysis. All chemicals of analytical grade were used as received without further processing.

1.2. Cell lines

The monkey kidney fibroblast cell line (Vero) was obtained from KCLB (Korean Cell Line Bank, Seoul, Korea) and were maintained in a media, Minimum essential eagle (MEM) alpha modification supplemented with 10% fetal bovine serum (FBS) (Hyclone, Thermo scientific, USA) and 1% penicillin/streptomycin (PPA, Cell culture company, Austria). The cells were maintained in 100 Φ cell culture dishes (SPL Lifesciences, Pocheon, Korea) in a humidified 5% CO₂ incubator at 37°C.

The Human articular chondrocyte cell line (HAC) were derived from the finger joint of a newborn baby and were maintained in Dulbecco's modified eagle's medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Thermo scientific, Australia), 1% Antibiotic-Antimycotic (Gibco, Life technologies, USA) and 2mM L-Glutamine (Gibco, Life technologies, USA). The cells were maintained in 175 cm² vented Flask BioCoat (Corning, USA) in a humidified 5% CO₂ incubator at 37°C.

1.3. Crosslinking Agents

The GTA (50% v/v) aqueous solution, DL-Gly powder ($\geq 90\%$), DMS and EDC both powder were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Scaffold Preparation Method

2.1. Preparation of porous gelatin scaffolds

The crosslinked-scaffolds were prepared by the method shown in Figure 1. This detailed method is described as follows [20, 35].

The gelatin solution was prepared by dissolving 10% w/v of gelatin (Type B, Sigma-Aldrich) in deionized water at 50°C with magnetic stirring for 20 minutes. This gelatin solution was poured into polystyrene molds such as petri dish, 12-well plate or others shapes depend of the assay's requirements.

Then the scaffolds were frozen at -20°C [20] for 2 hours and were lyophilized using Freeze Dryer Machine (Model SFDSM24S, Samwon, South Korea) for 24 hours without rinsing since no residual chemical needs to be removed. After freeze-drying, porous scaffolds were formed and it was immersed into the different crosslinking solutions.

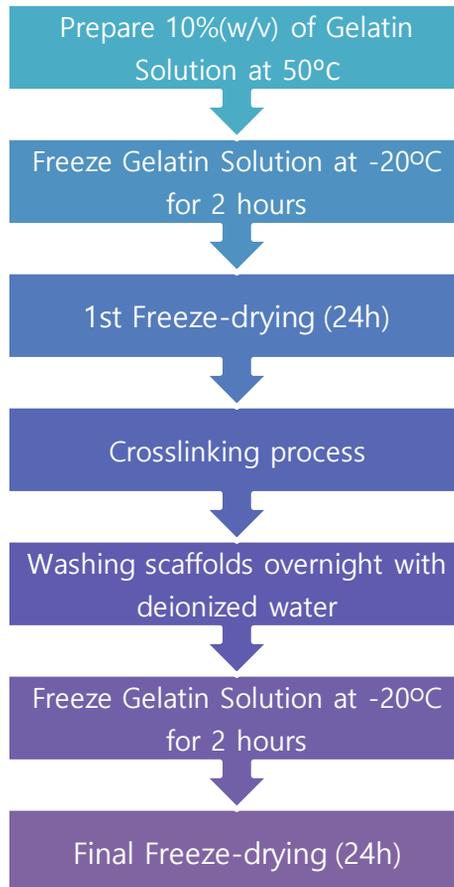


Figure 1. Schematic illustration of the fabrication method of the porous crosslinked scaffolds.

2.2. Crosslinking conditions

Several crosslinking methods were examined in this study: GTA (50% v/v) aqueous solution, DL-Gly powder ($\geq 90\%$), EDC and DMS. The dried gelatin scaffolds were crosslinked by treating them with different reagents at their respective optimal conditions summarized in Table 1. The crosslinking reaction schemes of EDC and GTA are shown in the Figure 2 [36, 42]. However, the mechanism of the crosslinking reaction between DL-Glyceraldehyde and amine groups of gelatin remains unknown [38], also it is just know that DMS not polymerize, and form crosslinks only with free amino groups separated by a distance equivalent to their respective molecular lengths [23].

GTA was used to crosslink the scaffold at a concentration of 0.50% v/v for 3 hours [36, 37]. DL-Gly was dissolved at a concentration of 0.50% w/v in 70% v/v ethanol/water, and the scaffolds were placed into this solution for 19 hours [38]. DMS was dissolved at a concentration of 1% w/v in 0.2M of Tris Buffer then the scaffolds were immersed into the DMS solution for 2 h [23, 39]. EDC was dissolved at a concentration of

0.1% w/v in 95% of ethanol for 24 hours [34]. All the crosslinking treatments were carried at room temperature.

After crosslinking for the described period of time, the membranes were washed extensively with deionized water overnight to remove traces of crosslinking agents, these steps contribute to the swelling of the scaffold. Then, the scaffold was put in the freezer at -20°C for 2 hours and then in a freeze-dryer machine for the second time for 24 hours [35, 23]. The scaffolds were stored at 4°C until used for further analysis.

Table 1. Optimal crosslinking conditions for porous gelatin scaffolds

Crosslinking method	Optimal conditions			References
	pH	Concentration	Time (h)	
GTA ^a	7.4	0.5% v/v	3	[36, 37]
DL-Gly ^b	7.0	0.5% w/v	19	[38]
DMS ^c	9.0	1.0% w/v	2	[23, 39]
EDC ^d	7.0	0.1% w/v	24	[34]

^aGTA was dissolved in deionized water. ^bDL-Gly was dissolved in 70% of ethanol. ^cDMS was dissolved in 0.2 M of Tris buffer. ^dEDC was dissolved in 95% of ethanol.

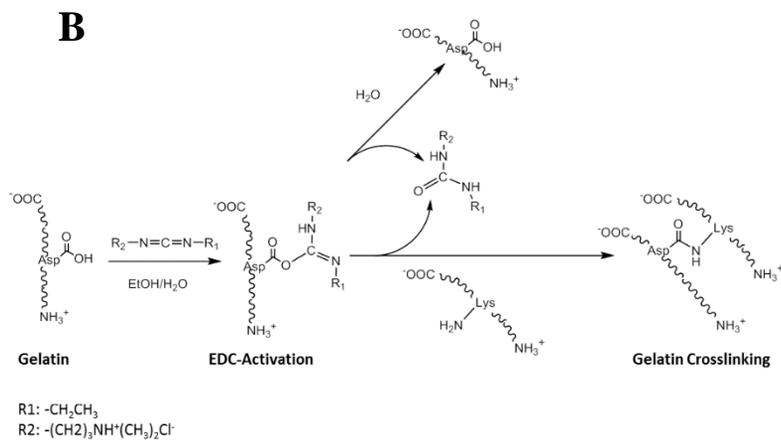
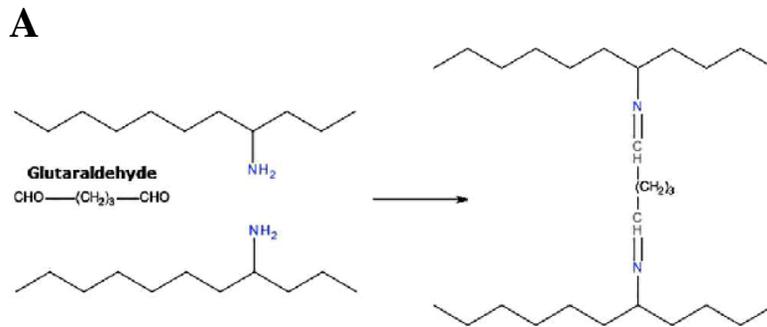


Figure 2. Crosslinking reaction schemes. (A) Schematic of the mechanism of the crosslinking reaction between gelatin and GTA and (B) crosslinking reaction between EDC and gelatin.

3. Characterization of the Scaffolds

3.1. Measurement of swelling ratio

The water absorption capacities of the gelatin scaffolds were determined by soaking them in Minimum essential eagle (MEM) alpha modification medium inside a humidified 5% CO₂ incubator at 37°C. After the designated soaking time had elapsed (1, 3, 6, 24 and 48h), the scaffold was wiped with filter paper to remove excess liquid and then weighted (W_t). The swelling ratio was calculated according to,

$$\text{Swelling ratio (\%)} = [(W_t - W_0)/W_0] \times 100$$

, where W_0 is the weight of the dried scaffold [35].

3.2. In vitro biodegradation assay

3.2.1. Collagenase degradation

Pieces of scaffolds of different weights were immersed in 5 ml of PBS at pH 7.4 containing 6 µg/ml of collagenase. After incubation in a humidified 5% CO₂ incubator at 37°C the loss of weight of scaffolds were monitored. Scaffolds were removed from the medium at different intervals (1, 3, 6 and 24 h), washed with distilled water and dried for 24 hours inside a laminar flow hood. The degree of degradation is expressed as percentage of weight loss (Degradation) as follows:

$$\text{Degradation (\%)} = (W_o - W_t)/W_o \times 100$$

, where W_o is the original weight and W_t is the weight at time t .

3.2.2. PBS degradation

Pieces of scaffolds of different weights were immersed in 5ml of PBS. After incubation in a humidified 5% CO₂ incubator at 37°C the loss of weight of scaffolds were monitored. Scaffolds were removed from the medium at different intervals (1, 3, 6, 24 h, 1 week and 2 weeks), washed with distilled water and dried for 24 hours inside a laminar flow hood. The degree of degradation is expressed as percentage of weight loss (Degradation) as follows:

$$\text{Degradation (\%)} = (W_o - W_t)/W_o \times 100$$

, where W_o is the original weight and W_t is the weight at time t .

3.3. Scanning electron microscopic examination

The morphology of the lyophilized scaffolds were examined by scanning electron microscopy (FE-SEM, Quanta 250 FEG, Japan) operating a 15 kV.

Also the cultured cells were analyzed using scanning electron microscopy. Previously, the scaffolds were fixed by immersion in 3% v/v of Glutaraldehyde at 4°C overnight, and then the gelatin samples were frozen at -20°C. Afterward, scaffolds were critically point dried using a Freeze Dryer machine for 24 hours [38, 40]. All samples were mounted using carbon tape on aluminum SEM stubs and were examined with magnifications from x500 to x3 000.

4. In vitro Cell Culture

4.1. Sterilization of the scaffolds

The scaffolds were sterilized in 70% ethyl alcohol and exposed to UV light for 24 hours; then, for removing the alcohol completely, they were soaked in PBS for 30 min 3 times, then soaked in culture medium for 30 min 3 times, and soaked in PBS again for 30 min 3 times [35].

For the swelling ratio and biodegradation analysis, the scaffolds were exposed only to UV light for 24 hours. All the above procedures were conducted in a Laminar-Flow Hood.

4.2. Cell Seeding

In this study two cell lines were tested, each one under specific conditions. The monkey kidney fibroblast cell line (Vero) were maintained in a media, Minimum essential eagle (MEM) alpha modification supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. These cells were maintained in 100 Φ cell culture dishes in a humidified 5% CO₂ incubator at 37°C.

The Human articular chondrocyte cell line (HAC) were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Antibiotic-Antimycotic and 2mM L-Glutamine. The cells were maintained in 175 cm² vented Flask BioCoat in a humidified 5% CO₂ incubator at 37°C.

In both cases the medium was changed every 2–3 days. When the cells reached confluence, they were removed from the culture dish using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich), centrifuged, and re-suspended in their respective medium of culture.

For this experiment the scaffolds were created with the shape of 12-well plate, using 2ml of 10% w/v of gelatin solution. All the scaffolds

were sterilized before the cell seeding following the instructions that were explained above.

Then cells before seeded were counted using a hemocytometer. The cells were added to the medium to be seeded onto the scaffolds at a concentration of 1×10^5 cells ml^{-1} . Each scaffold was seeded with 1 ml of culture medium containing the respective cell line. The cell suspension was added to the scaffolds slowly in the middle of the scaffolds to allow cells to attach to the scaffold instead of sliding off to the side of the well. The scaffolds then were placed in the incubator at 37°C and 5% CO₂ and cells were allowed to grow for 6 days, the medium was changed every 3 days [38].

4.3. Cytotoxicity analysis by CCK-8 assay

An enhanced cell viability assay kit-8 (CCK-8, DogenBio Co., Korea) was used to measure cell viability and cell concentration according to the manufacturer's instruction. Briefly, the media of each sample was removed, rinsed with PBS, and add the same volume of fresh media to each well prior to adding CCK-8, to reduce material interference. Then 100 μ L of CCK-8 stock solution was added to each well and was incubated for 2 h in a humidified 5% CO₂ atmosphere. Cell proliferation and viability was determined by reading the absorbance at 450 nm (optical density, OD₄₅₀) using a multiplate reader (SpectraMAX I3x, MinimaxTM 300 imaging cytometer). The results were normalized to the negative control (just scaffold with medium without cells). All the assays were performed in triplicates.

Results and Discussion

1. Characterization of the scaffolds

In the next page Figure 2 shows the microstructure of the porous gelatin scaffolds, the scanning electron microscopic (SEM) images shows the samples of the non-crosslinked scaffold and scaffolds crosslinked with GTA, DL-Gly, DMS and EDC solutions. In particular, the structure in the non-crosslinked samples appeared to have collapsed with less interconnectivity with an average pore size of about 20–236 μm and pore walls are much thinner compared with the other samples.

On the other hand, the crosslinked structures showed more pore interconnectivity, varying of the crosslinking agent used in each membrane. The cross-section morphology indicates that the scaffolds crosslinked with GTA appear in the interconnected network structure with an average pore size of about 20–100 μm (Figure 2C), DI-Gly with an average of 25-90 μm (Figure 2D), DMS and EDC with an average of about 40-650 μm (Figure 2E) and 10-45 μm (Figure 2F), respectively.

The thickness of the pore walls of the EDC crosslinked samples are the thinnest compared with the other crosslinked samples and although the amount of the pores is much higher, these both characteristics conferred less stability to this scaffold.

The most interconnected and stable membrane is the sample crosslinked with DL-Gly that is correlated with the good performance under cell culture experiments.

Thereby, all the microstructure of the crosslinked scaffolds showed interconnectivity, which is critical for cell migration and proliferation and facilitating the exchange of nutrients and waste products [36].

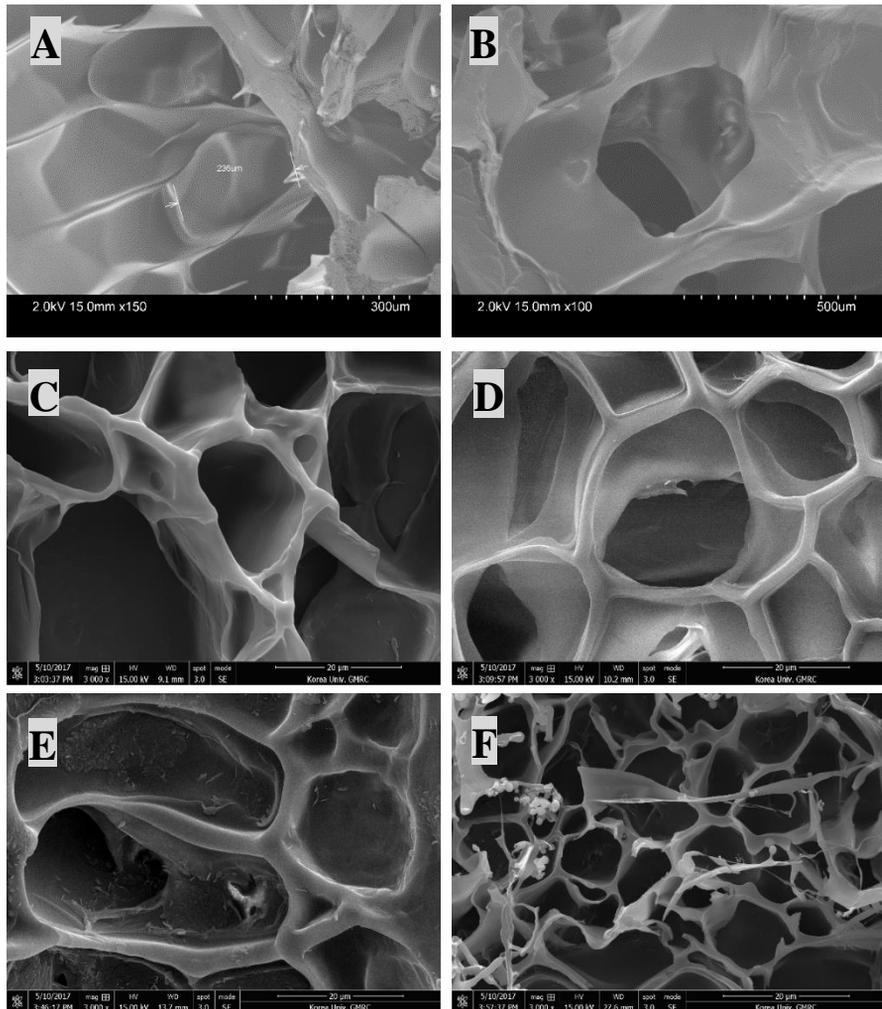


Figure 3. SEM images showing interconnectivity and the pore structure of gelatin scaffolds: non-crosslinked and with the different crosslinking agents. (A), (B) non-crosslinked samples both at 150× and 100× magnification, respectively. (C) Scaffold crosslinked with GTA solution, at 3000× magnification. (D) Scaffold crosslinked with DL-Gly, at 3000× magnification. (E) Scaffold crosslinked with DMS and (F) with EDC, both at 3000× magnification. Scale bars, (A) 500 μm, (B) 500 μm and (C, D, E, F) 20 μm.

2. Swelling ratio of the scaffold samples

Swelling ratios of the crosslinked porous gelatin scaffolds at determinate times are presented in Figure 3. The water absorption capability of gelatin in the scaffold is limited by the 3D structure or the wall thickness of the scaffold [35]. For this reason, a comparison of the swelling ratio of all the gelatin samples crosslinked at their respective optimal conditions reveals that DL-Gly causes a maximum increment in the swelling ratio of gelatin scaffold, since the mechanical strength of the DL-Gly crosslinked scaffolds are higher these samples have more capability of water absorption compare with the other samples.

Furthermore, as well as the good efficiency of GTA as a crosslinker is widely known, it also showed a high swelling ratio percentage. DMS also showed good swelling ratio properties, however, EDC loosed weight after the first 24 hours of incubation at 37°C due to its low resistance to that temperature incubation and the samples began to degrade.

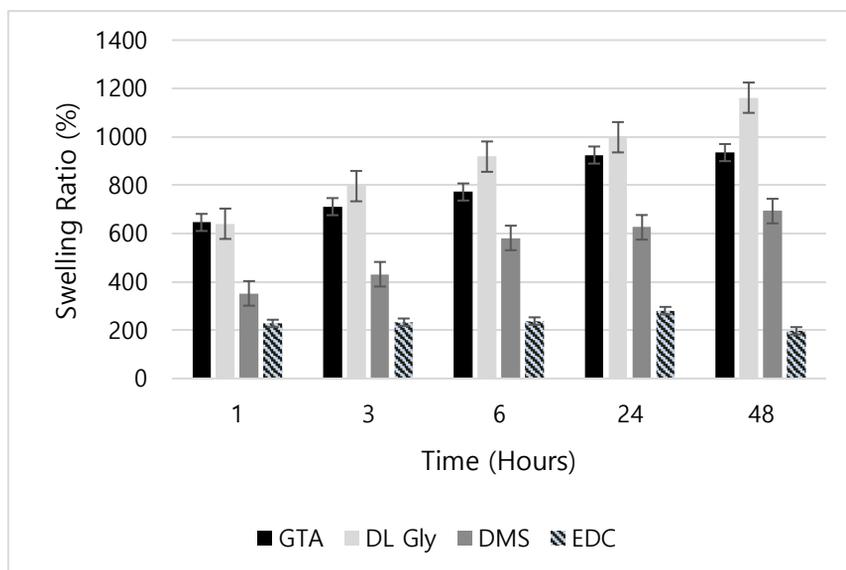


Figure 4. Swelling ratio percentages of crosslinked gelatin membranes with the different crosslinking agents. Percentages of water absorbed of porous gelatin membranes in cell culture medium at 37°C, comparing the final weight with the initial dry weight. The assay was performed in triplicates.

3. *In vitro* biodegradability

3.1. *In vitro* Collagenase degradation

The susceptibility of the crosslinked porous gelatin scaffolds to bacterial collagenase degradation was assessed. During *in vitro* digestion experiments, gelatin samples were exposed to bacterial collagenase for different time periods and it was found that complete degradation of crosslinked gelatin samples occurred at 24 hours of incubation at a concentration of 6 $\mu\text{g/ml}$. Figure 4 illustrates the fact that the gelatin membranes crosslinked by all the four methods were not resistant to digestion by collagenase. Among the crosslinked samples, gelatin crosslinked with EDC degraded at a much faster rate compared to the other samples and was almost completely degraded after 3 hours of incubation (97.4%). Solubilization was much lesser for gelatin membranes crosslinked with GTA, however, the general rate of degradation is very fast because all the crosslinked gelatin scaffolds were degraded before the 24 hours of incubation.

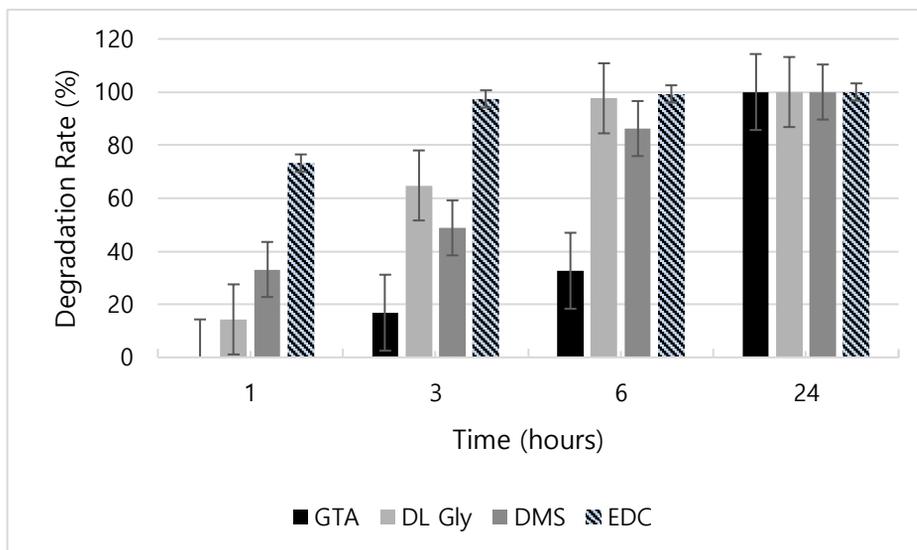


Figure 5. Results of *in vitro* biodegradation analysis using collagenase enzyme solution. Samples were incubated at 37°C, the final dry weight were compared with the initial weight of the lyophilized sample. The assay was performed in triplicates.

3.2. *In vitro* PBS degradation

The results obtained from the degradation of porous scaffold tested using PBS are shown in Figure 5. All the gelatin scaffolds remained for the entire two weeks test. PBS was used like a control to compare with the enzymatic degradation of collagenase.

However, even using PBS all the crosslinked scaffolds had a high ratio of degradation; except GTA which showed a slow degradation rate, because after 2 weeks of incubation just the 24.6% of the samples were degraded. Among the crosslinked samples, EDC-treated gelatin scaffolds degraded at a much faster rate compared to the other samples, after 2 weeks, 94.2% were degraded. The rate of degradation is also an indirect indicator to know the crosslinking degree and therefore the stability and tensile strengths of the crosslinked scaffolds [6].

It is shown that both scaffolds in PBS solution or collagenase enzyme showed mass loss with increasing incubation time, however the weight loss ratio in collagenase was significant higher compared with the scaffolds in PBS solution until 24 hours.

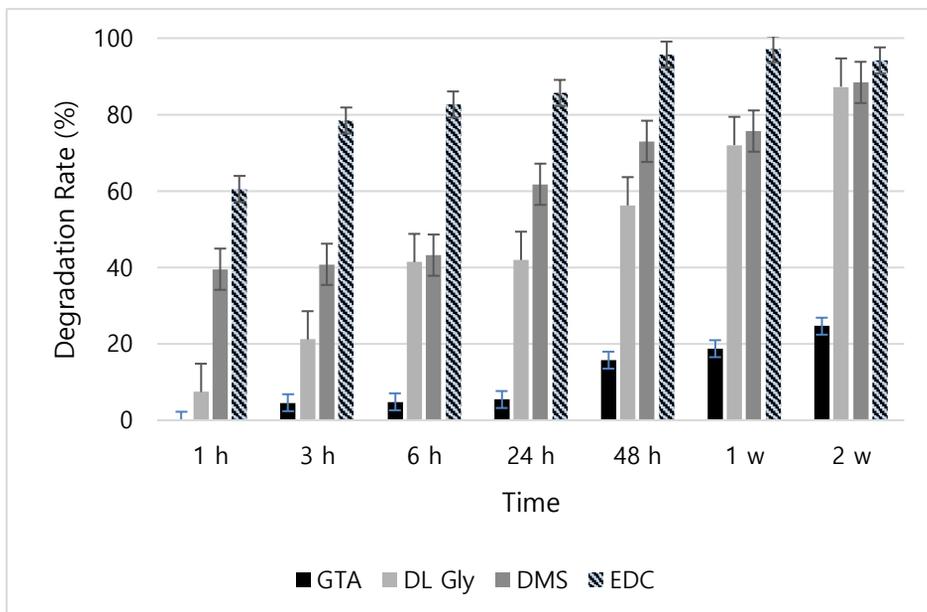


Figure 6. Results of *in vitro* biodegradation analysis using PBS solution. Samples were incubated at 37°C, the final dry weight were compared with the initial weight of the lyophilized sample. The assay was performed in triplicates.

4. Cytotoxicity evaluation

4.1. Cellular viability

4.1.1. Human articular chondrocyte (HAC)

To analyze the biocompatibility of the crosslinking agents the cell viability were tested using direct contact assays. The scaffold samples were incubated with HAC cells, at an initial density of 1×10^5 cells ml^{-1} in 12-well plates adding 1 ml of cell suspension per well, in a humidified 5% CO_2 incubator at 37°C for 3 days. After the incubate period, the cell activity was read using CCK-8 kit, the plate was incubated with 100 μl of CCK-8 solution for 2 hours and the formazan dye produced by viable cells was quantified by a spectrophotometer by measuring the absorbance of the dye solution at 450 nm.

The viability of the groups treated with GTA and DL-Gly was significantly well compared with the other 2 groups who showed a viability percentage below 40% [Fig. 6]. Compared with the GTA

crosslinked scaffolds the DL-Gly group showed higher viability percentage (96.8%).

Therefore, these results confirmed that the samples crosslinked with DL-Gly showed a reduced toxicity relative to GTA reagent and could be a good alternative to overcome the restriction of using GTA as a crosslinking agent in the pharmaceutical field.

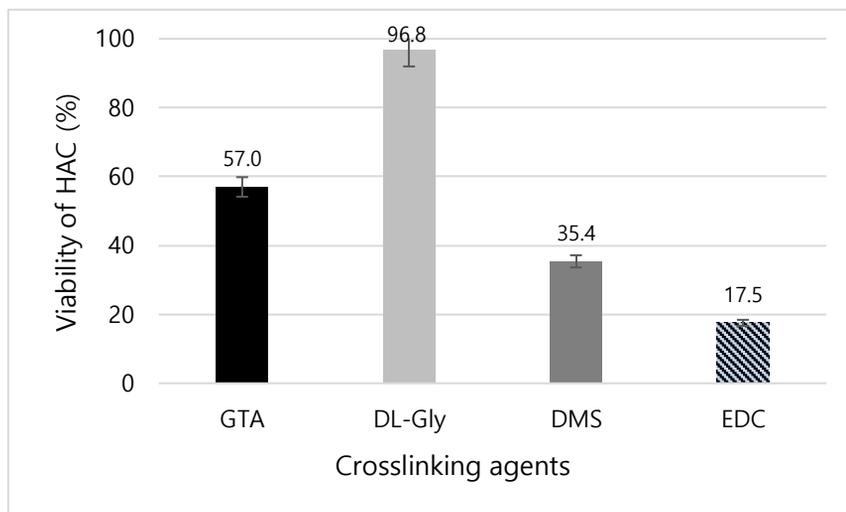


Figure 7. Cytotoxic effects of crosslinking reagents on human articular chondrocyte (HAC) cells after 3 days of culture on the respective scaffolds. Cell viability was determined using CCK-8 assay and expressed as percentages of control, which was culture without any kind of scaffolds. Data are expressed as mean values \pm standard deviation of independent experiments, all the assays were performed in triplicates.

4.1.2. Monkey kidney fibroblast (Vero)

The biocompatibility of the scaffolds incubated with Vero cells were analyzed also using direct contact assays, and the cell activity was measured with CCK-8 kit following the same protocol explained above. The initial density was 1×10^5 cells ml^{-1} in 12-well plates adding 1 ml of cell suspension per well, in a humidified 5% CO_2 incubator at 37°C , and the absorbance of the samples was read after 3 days of incubation. The results are shown in the Figure 7.

Vero cells are one of the more commonly used mammalian continuous cell lines in microbiology, and molecular and cell biology research, because of its low requirements of cultivation and continuous propagation [41]. For this reason the viability percentage of all the samples groups are above 50%.

However, EDC method showed the same low performance with Vero cells as with HAC cells (51.8%). The cell viability of GTA is above 80% which demonstrated a low cytotoxicity effects, these results could be due to the low concentration used to crosslink the gelatin scaffold and

also pre-washing steps before cell seeding, which reduced the toxic products released by the GTA.

The viability percentage of gelatin scaffolds crosslinked with DL-Gly is almost 80%, these results suggest that DL-Gly samples are well tolerated *in vitro* and could result in a biocompatible and non-toxic crosslinking agent. Nonetheless, the mechanism of the crosslinking reaction between the glyceraldehyde and the gelatin remains unknown and needs more studies.

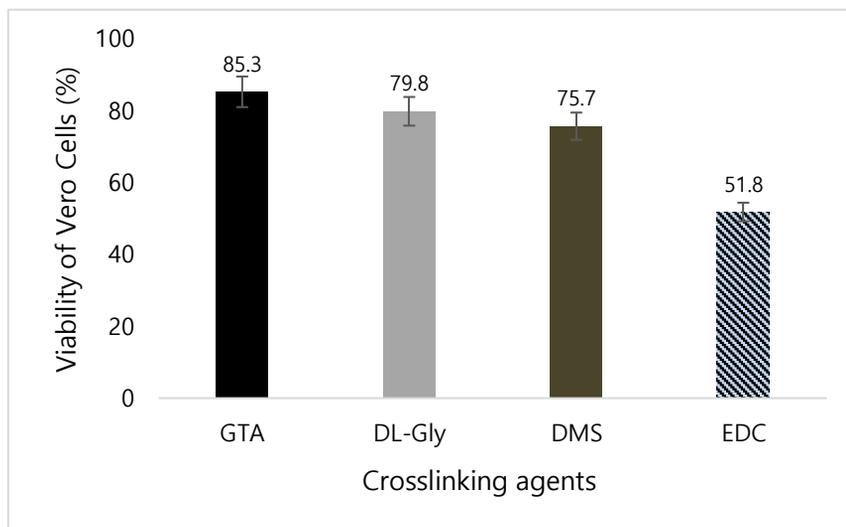


Figure 8. Cytotoxic effects of crosslinking reagents on Vero cells after 3 days of culture on the respective scaffolds. Cell viability was determined using CCK-8 assay and expressed as percentages of control, which was culture without any kind of scaffolds. Data are expressed as mean values \pm standard deviation of independent experiments, all the assays were performed in triplicates.

4.2. Cellular Proliferation

4.2.1. Human articular chondrocyte (HAC) growth kinetics

At 6 days, after seeding the cells on the scaffolds at an initial density of 1×10^5 cell ml^{-1} , the cell proliferation was measured by the absorbance change from the CCK-8 kit and by estimating the number of HAC cells using a microplate reader. As indicated by the estimated cell concentration values of Figure 8.

The cell proliferation results on day 6 for the DL-Gly crosslinked scaffold shows higher concentration number (19.3×10^5 cells ml^{-1}) compared with all the others crosslinking reagent groups. The groups DMS and EDC crosslinked scaffolds showed similar results, with very low cell proliferation.

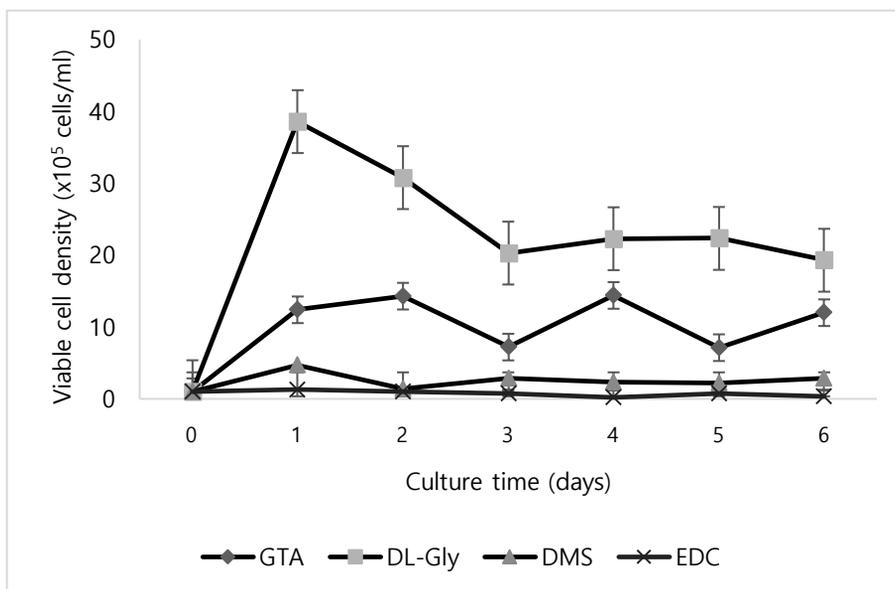


Figure 9. Proliferation of HAC cells in the crosslinked gelatin scaffolds. The Growth of HAC cells was analyzed using CCK-8 assay for 6 days of culture on the respective scaffolds at a 37°C.

4.2.2. Monkey kidney fibroblast (Vero) growth kinetics

In vitro cell culture experiments were performed seeding Vero cells in the scaffolds at an initial density of 1×10^5 cell ml^{-1} , for 6 days and the cell proliferation was measured using the CCK-8 kit and by estimating the number of Vero cells using a microplate reader. As indicated by the estimated cell concentration values in Figure 9A, all the data was evaluated daily.

In the scaffolds treated with GTA and DL-Gly, the cells grow quite well, and the number of cells of both groups are very similar after the 6 days of culture. However, the cell growth in the DMS and EDC crosslinked scaffolds is quite poor during the 6 days of cultivation, it is correlated with the increase of degradation of these scaffolds through the passage of days.

In Figure 9B, it can be seen that cells grown on each of the different scaffolds crosslinked with GTA and DL-Gly had better cell proliferation compared with the cells grown on the two-dimensional culture in polystyrene wells on day 6. This indicate that the cells on the

crosslinked scaffolds of these 2 groups could migrate into the pores of the scaffold thus allowing for more proliferation because the cells have more area to grow in a 3D environment.

However, after the 6 days of incubation the DMS and EDC crosslinked scaffolds showed an instable structure because more than 50% of the structure was degraded. Therefore, this is in agreement with the results of the low cellular concentration.

SEM analysis demonstrated the porous structure of the different crosslinked scaffold samples after being cultured with Vero cells for 4 days. The Figure 10 showed that the cells had successfully adhered on the surface and other cells have infiltrated into the pores of the gelatin-based biodegradable scaffolds.

The left side of the figure shows the porous surface of the cultured scaffolds at 500 x magnification, some cells have penetrated and filled the porous and other are distributed on the surface. On the right side of the image, the morphology of the cells attached on the scaffolds can be appreciated at 3000 x magnification.

The low cell proliferation of the cells in the EDC crosslinked scaffold is in agreement with the large number of empty pores (Figure. 10G, H). The morphology of the cells is also other parameter to be analyzed, only the GTA crosslinked scaffold have showed attached cells with damaged surface (Figure 10B) while the other groups exhibit smooth surface morphology (Figure 10D, F).

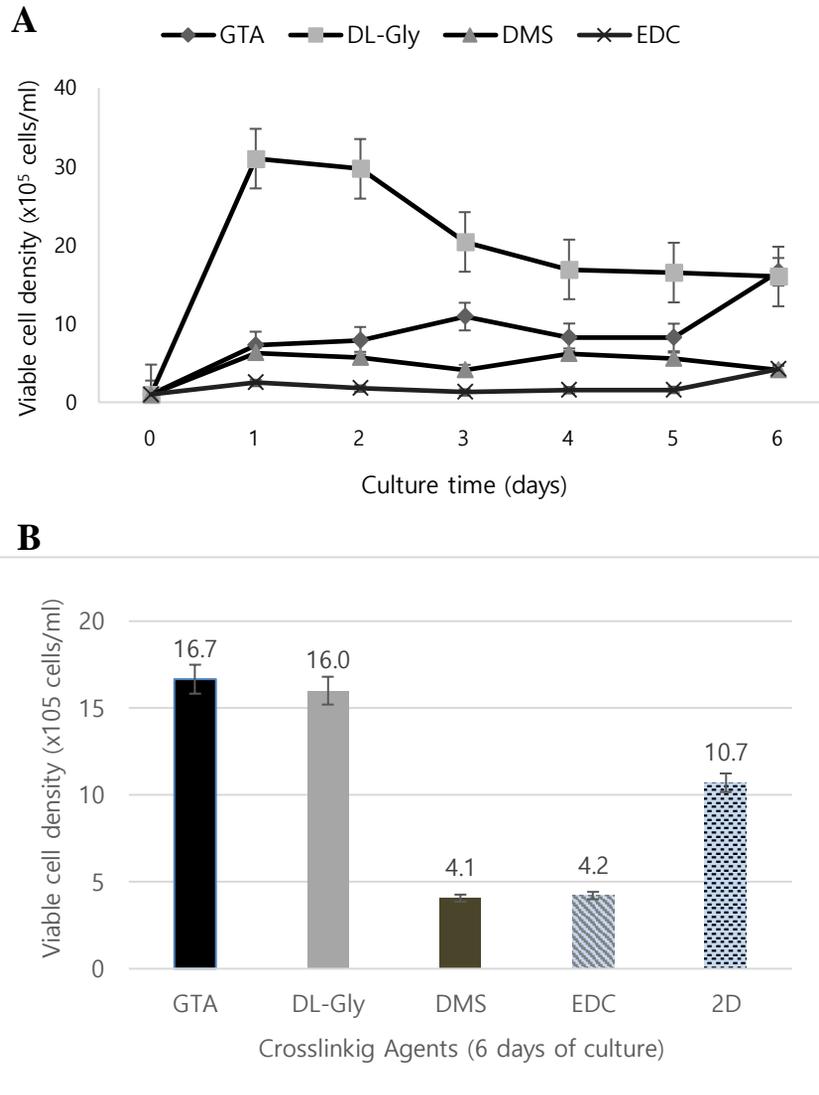


Figure 10. Proliferation of Vero cells in the crosslinked gelatin scaffolds. (A) The Growth of Vero cells was analyzed using CCK-8 assay for 6 days of culture period on the respective scaffolds at a 37°C. (B) Comparison of the viable cell density of crosslinked scaffolds against monolayer (2D) cell culture as a control on the day 6.

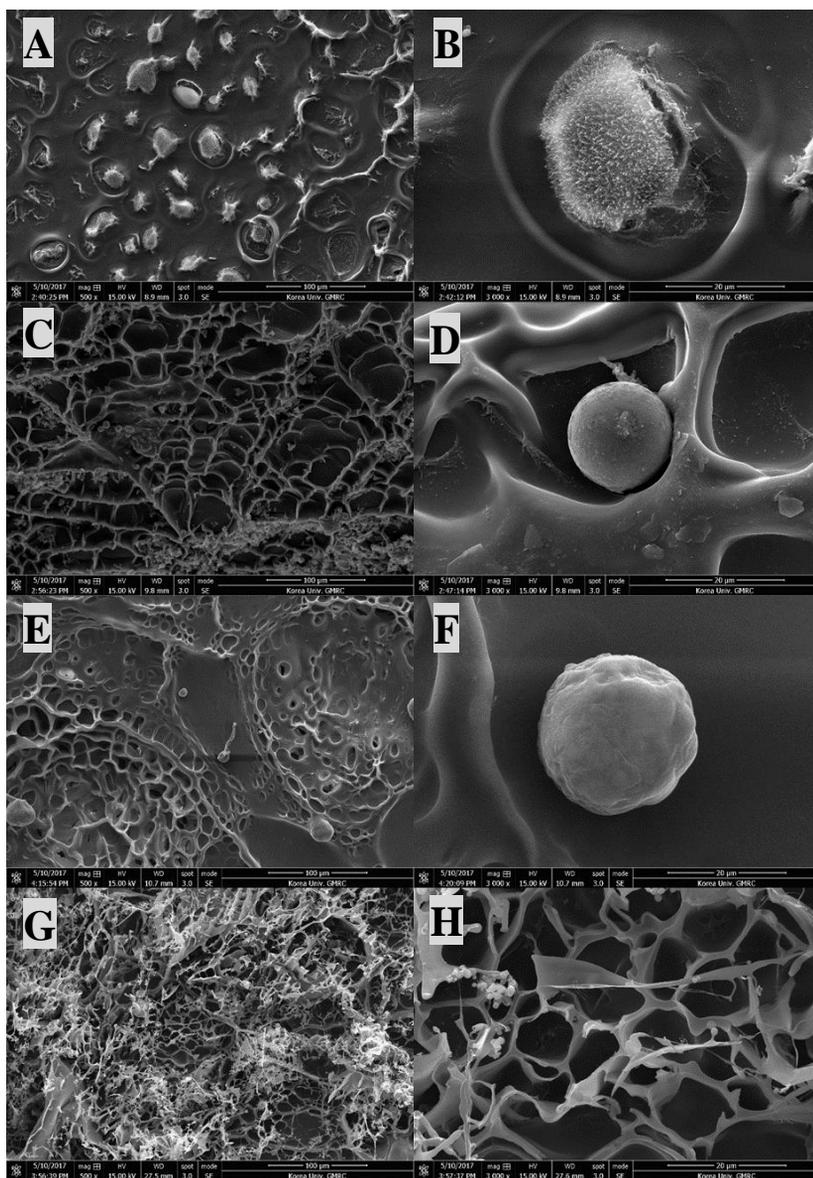


Figure 11. SEM micrographs of crosslinked gelatin porous scaffold seeded with Vero cells on day 4. (A, B) Scaffold crosslinked with GTA, (C, D) Scaffold crosslinked with DL-Gly, (E, F) Scaffold crosslinked with DMS and (G, H) with EDC. (A, C, E, G) SEM photos captured at 500x, scale bars 100um; and (B, D, F, H) captured at 3000x, scale bars 20um.

Conclusion

In conclusion, a variety of biological scaffolds have been prepared with defined biochemical and morphological characteristics. The methodology applied may be instrumental to produce specific scaffolds for tissue engineering. From this study, it was determined that the use of EDC is not a viable method for cross-linking durable gelatin scaffolds because the scaffolds degraded after only few days in cell culture medium at 37°C. Moreover, these results have shown promising potential of using gelatin-based matrices crosslinked with DL-Gly in cell culture lines and tissue engineering as a better alternative of using GTA because of its cytotoxicity

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Abstract in Korean

젤라틴기반의 바이오소재/생체 적합 물질의 물리적 및 생물학적 특성은 가교 방법에 의해 엄청난 영향을 받는다. 본 연구의 목적은 최적화된 세포 배양 라인에 안정적인 다공성 젤라틴 지지체를 만들기 위한 다양한 가교 치료법의 영향을 조사하는 것이다. 다공성 젤라틴 지지체를 가교 결합하기 위하여 여러 가지 다양한 방법들을 사용되었는데, glutaraldehyde (GTA), DL-glyceraldehyde (DL-Gly), dimethyl suberimidate dihydrochloride (DMS) 뿐만 아니라 N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)도 사용되었다. 가교 결합된 매트릭스의 물리적 및 생물학적 특성을 평가하기 위하여 팽창률, 구조 형태, 콜라겐 분해효소에 대한 저항성 및 PBS 소화능력, 그리고 시험관 내 세포 배양이 수행되었다. GTA가 세포 독성이 있기 때문에 부작용을 피하기 위한 대책으로 다른 가교제를 발굴하였다. 하지만, 단 몇 시간 후에 37°C에 있는 콜라겐 분해효소가 포함된 액체배지에서 모든 가교 결합된 지지체가 분해되었다.

게다가, vero 세포와 HAC 세포 계는 각각의 가교 결합된 지지체 상에서 자랐고, 세포 증식 분석에서는 세포의 농도와 세포의 생존 능력이 전통적인 배양방법인 단층 폴리스티렌 배양보다도 GTA와 DL-Gly로 가교 결합된 지지체 상에서 똑같거나 더 낫다는 것을 보여 주었다. 이러한 결과를 바탕으로, 낮은 독성, 건강한 세포 증식과 세포 생존뿐만 아니라 37°C에서 2주넘게 세포 배양 배지에서 가교 결합된 지지체의 용해 저항력이 있기 때문에, DL-Gly와 가교 결합된 젤라틴기반의 매트릭스 사용의 잠재적 가능성을 확인할 수 있었다.