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# Crosslinking of the electrospun gelatin nanofibers

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**Abstract**

Gelatin (Gt) nanofibers have been prepared by using an electrospinning process in a previous study. In order to improve their water-resistant ability and thermomechnical performance for potential biomedical applications, in this article, the electrospun gelatin nanofibers were crosslinked with saturated glutaraldehyde (GTA) vapor at room temperature. An exposure of this nanofibrous material in the GTA vapor for 3 days generated a crosslinking extent sufficient to preserve the fibrous morphology tested by soaking in 37 °C warm water. On the other hand, the crosslinking also led to improved thermostability and substantial enhancement in mechanical properties. The denaturation temperature corresponding to the helix to coil transition of the air-dried samples increased by about 11 °C and the tensile strength and modulus were nearly 10 times higher than those of the as-electrospun gelatin fibers. Furthermore, cytotoxicity was evaluated based on a cell proliferation study by culturing human dermal fibroblasts (HDFs) on the crosslinked gelatin fibrous scaffolds for 1, 3, 5 and 7 days. It was found cell expansion took place and almost linearly increased during the course of whole period of the cell culture. The initial inhibition of cell expansion on the crosslinked gelatin fibrous substrate suggested some cytotoxic effect of the residual GTA on the cells.


*Keywords:* Gelatin; Electrospinning; Crosslinking

## 1. Introduction

Gelatin is a protein biopolymer derived from partial hydrolysis of native collagens, which are the most abundant structural proteins found in the animal body of skin, tendon, cartilage and bone [1]. Due to a wealth of merits such as biological origin, nonimmunogenicity, biodegradability, biocompatibility, and commercial availability at relatively low cost, gelatin has been widely used in the pharmaceutical and medical fields as sealants for vascular prostheses [2–4], carrier for drug delivery [5–7], dressings for wound healing [8,9], and so forth.

It is well known that gelatin is good film-forming and widely utilized in the form of films. However, this material is poor in fiber processing, thus practical microfibers of gelatin via a conventional wet/dry spinning are not common [10]. Recently, this type of biopolymer has been successfully spun into nanoscale fibers through a ultrafine fiber manufacturing technology called electrospinning [11,12]. With electrospinning, nanoscale gelatin fibers were conveniently obtained by employing a fluorinated alcohol of trifluoroethanol (TFE) as a solvent [13]. Nanofibers are very promising candidates in a variety of applications [12], especially in the situations where surface area is highly concerned. In the biomedical field, gelatin nanofibers can be potentially useful in developing biomimicking artificial extracellular matrix (ECM) for engineering tissues, dressings for wound healing, and drug releases. However, the as-electrospun nanofibrous structure of gelatin is water soluble and mechanically weak. This can limit its applications. For a long-term biomedical application, an electrospun gelatin nanofibrous membrane must be crosslinked as done on its film counterparts. Crosslinking treatment would be able to improve both water-resistant ability and thermomechanical performance of the resulting nanofibrous membranes.

In the literature, several physical and chemical methods have been reported for crosslinking collagenous materials. Physical methods include dehydrothermal treatment and UV-irradiation [14,15], however, they are generally less efficient.

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Many chemicals such as formaldehyde, glutaraldehyde, carbodiimide and dextran dialdehyde, have been used to chemically modify gelatin for biomedical applications. Amongst, glutaraldehyde (GTA) is by far the most widely used chemical, due to its high efficiency in stabilizing collagenous materials [16]. GTA based crosslinking of collagenous materials significantly reduces biodegradation, making the materials biocompatible and nonthrombogenic while preserving biological integrity, strength and flexibility. GTA is also easily available, inexpensive and capable of accomplishing the crosslinking in a relatively short time period. Although other crosslinking agents were reported to reduce cytotoxicity, they cannot match GTA in collagen stabilization [17]. The risk of cytotoxicity can be improved by lowering the concentration of GTA solutions [18] or thorough treatment prior to usage.

The objective of this study is to make the as-electrospun gelatin nanofibers water insoluble through a GTA crosslinking treatment so as to preserve their fibrous morphology and enhance their thermal and mechanical performance. The crosslinking was carried out in a saturated GTA vapor as described in Ref. [19]. The thus crosslinked gelatin fibrous membranes were characterized through dissolution test, DSC thermal analysis, tensile test, and in vitro cellular cytotoxicity experiment.

## 2. Materials and methods

### 2.1. Materials

Polymers of gelatin type A (Approx. 300 Bloom, Sigma, MO, USA) from porcine skin in powder form, and solvent of 2,2,2-trifluoroethanol (TFE) (purity $\geq$ 99.0%, Fluka, Buchs, Switzerland) were obtained from Sigma–Aldrich (St Louis, MO, USA). Crosslinking agent of aqueous GTA solution (25%) was a product of Merck (Hohenbrunn, Germany). They were used as received without further purification. A transparent gelatin/TFE solution (10% w/v) for electrospinning was prepared by dissolving gelatin in TFE that was stirred at room temperature up to 6 h.

### 2.2. Electrospinning

Preparation of electrospun gelatin nanofibers from a laboratory electrospinning setup has been reported in our previous study [13]. Briefly, the gelatin/TFE solution held in a 5 ml syringe was delivered into a blunted medical needle spinneret (OD 1.2 mm, ID 0.84 mm) through a Teflon tubing by a syringe pump (KD-100, KD Scientific, Inc., USA). A polarity reversible high voltage power supply (RR50-1.25R/230/DDPM, Gamma High Voltage Research, USA) was used to charge the spinning dope of gelatin/TFE by directly clamping one electrode to the metal needle spinneret, and another to an aluminum foil wrapped on a lab rack. The separating distance between the needle tip and the aluminum foil was set to 13 cm. Other operating parameters in a chamber for producing the gelatin nanofibers are as follows: voltage 10 kV, flow rate 0.8 ml/h, ambient temperature 21.5 °C, and humidity 75%. The obtained nanofibrous membranes were stored in a vacuum oven for 24 h to remove residual solvent and then were transferred into a dry cabinet for storage at room temperature.

### 2.3. GTA vapor crosslinking

The crosslinking process was carried out by placing the air-dried gelatin nanofibrous membrane ($\sim$0.1 mm thick) together with a supporting aluminum foil in a sealed dessicator containing 10 ml of aqueous glutaraldehyde solution in a Petri dish [19]. The membranes were placed on a holed ceramic shelf in the dessicator and were crosslinked in the glutaraldehyde vapor at room temperature. An optimized extent of crosslinking was determined by testing the dissolubility of those crosslinked gelatin nanofibrous membranes immersed in 37.0 °C de-ionized water for varied time periods. Samples with distinct crosslinking extents were prepared by exposing the nanofibrous membranes in the GTA vapor for a different time course. After crosslinking, the samples were exposed in a fume hood for 2 h followed by a post-treatment at 100 °C for 1 h to remove residual GTA and partially enhance the crosslinking [20].

### 2.4. Dissolvability test

The crosslinked gelatin nanofibrous membranes were cut into a size of $2\times2$ cm$^2$ and immersed into warm DI water (37.0 °C) for certain period of time to test their dissolvability. This experimental condition was selected in order to simulate a real situation of gelatin nanofibers in physiological applications such as for tissue engineering scaffolds or release carriers.

### 2.5. Characterizations

The electrospun nanofiber morphology was observed under a field emission scanning electronic microscope (FESEM) using a Quanta FEG 200 machine (FEI Company, The Netherlands) operated at an acceleration voltage of 10 kV. Prior to FESEM, samples were sputter coated for 90 s with gold using a JEOL JFC-1200 fine coater. Based on the SEM photos, fiber diameters of the nanofibrous membranes were analyzed using an image visualization software ImageJ developed by Upper Austria University of Applied Sciences.

Thermal properties of the electrospun fibers were measured by a TA Instruments 2920 Differential Scanning Calorimeter (DSC). The instrument was calibrated with an Indium standard, and a nitrogen atmosphere (flow rate = 50 ml/min) was used throughout. All samples were quenched to $-60$ °C with liquid nitrogen before starting heating runs, then heating was intrigued at 10 °C/min to 220 °C. The thermograms recorded were analyzed by TA Universal Analysis 2000 software.

Tensile properties of the electrospun fibrous membranes were determined with a tabletop MicroTester (Instron 5845, USA) using a low force load cell of 10N capacity. Strip-shaped specimens ($30\times6$ mm$^2$) were tested at a crosshead speed of

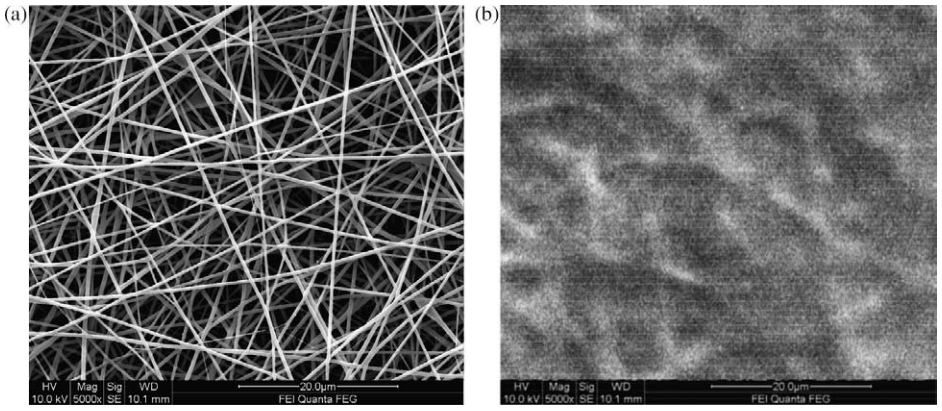


Fig. 1. Morphologies of gelatin nanofibers: (a) from electrospinning of a 10% w/v gelatin/TFE, and (b) the smeared surface layer of gelatin nanofibrous membrane after adding a drop of water.

10 mm/min. The ambient condition was controlled to be 24.7 °C and 74% humidity. Thickness of the specimens for the as-electrospun and crosslinked Gt fibrous samples was around 100 and 25 μm, respectively, which were measured with a digital micrometer having a precision of 1 μm. Ultimate strength, Young's modulus as well as tensile elongation were calculated based on the generated tensile stress–strain curves.

### 2.6. Cytotoxicity evaluation

The cytotoxicity of crosslinked nanofibrous gelatin membranes was evaluated based on a fibroblast proliferation study. The fibroblasts used were a gift from the National University Hospital, Singapore. The human normal skin was obtained by surgical removal under local anesthesia after informed consent. Epidermis and subdermal fat were removed from sterile biopsies of the normal skin. The specimens were minced into pieces of 1–2 $mm^3$ in sterile tissue culture dishes and gently overlaid with DMEM supplemented with 10% FBS with antibiotics. Explants were incubated at 37 °C in a humidified $CO_2$ incubator for 10 days and fed every 3 days and fibroblasts were harvested from primary cultures by trypsin–EDTA treatment and replated. The human dermal fibroblast cultures at 2–4 passages were used for this study.

Prior to cell seeding, the samples were sterilized under UV for 3 h, followed by a soaking in PBS for 24 h with five changes and a culture medium soaking for 1 day. Then, the human dermal fibroblasts were seeded ($2\times10^4$ cells/$cm^2$) on the crosslinked gelatin nanofibrous matrices in 24 well tissue culture plates (TCPS). Nanofibrous scaffolds of PCL, gelatin/PCL blend, and TCP substrate were used as controls. The cell proliferation was monitored for 1, 3, 5, and 7 days ($n=6$ for each time point per group) by MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt). In order to monitor cell proliferations on different substrates, the number of cells was determined by using the colorimetric MTS assay (CellTiter 96® $Aq_{ueous}$ Assay). The mechanism behind this assay is that metabolically active cells react with tetrozolium salt in the MTS reagent to produce soluble formazan dye that can be observed at 490 nm. The cellular constructs were rinsed with PBS followed by incubation with 20% MTS reagent in serum free medium for 3 h. Thereafter, aliquots were pipetted into 96 well plates and the samples were read using the spectrophotometric plate reader (FLUOstar OPTIMA, BMG Lab Technologies, Germany) at 490 nm.

## 3. Results and discussion

### 3.1. Fiber morphologies before and after crosslinking

As reported earlier [13], electrospinning of 10% w/v gelatin/TFE solutions gave rise to beads-free and randomly arrayed ultrafine fibrous nonwovens with averaged fiber diameters around 200–300 nm (Fig. 1(a)). Since gelatin is water soluble, even a drop of water on the membranes can immediately destroy the nanofibrous structure as shown in Fig. 1(b). Another character is that the electrospun fibers are even able to gradually form point bonds at the fiber junctions if placed in a high humidity ambient, e.g. 80–90% for a certain period of time. Due to the sensitivity to water contact or high humidity, conventional crosslinking approach of immersing cast gelatin films into aqueous GTA solution is not feasible for crosslinking the present nanoscale thin gelatin fibers. By placing the nanofibrous gelatin into a dessicator filled with saturated GTA vapor, the gelatin nanofibers could be reasonably crosslinked. Crosslinking of collagenous materials with GTA involves the reaction of free amino groups of lysine or hydroxylysine amino acid residues of the polypeptide chains with the aldehyde groups of GTA [21]. After GTA vapor crosslinking, the membranes became visibly yellowish and slightly shrunk

Table 1
Dissolvability test to determine optimized crosslinking extent

| Immersion time at 37.0 °C | Time length of crosslinking[a] | | | | | |
|---|---|---|---|---|---|---|
| | 6 h | 12 h | 1 day | 2 days | 3 days | 4 days |
| Day 1 | Y | Y | Y | Y | Y | Y |
| Day 2 | | Y | Y | Y | Y | Y |
| Day 3 | | | | Y | Y | Y |

[a] 'Y' denotes membrane samples remain after the specified period of soaking.

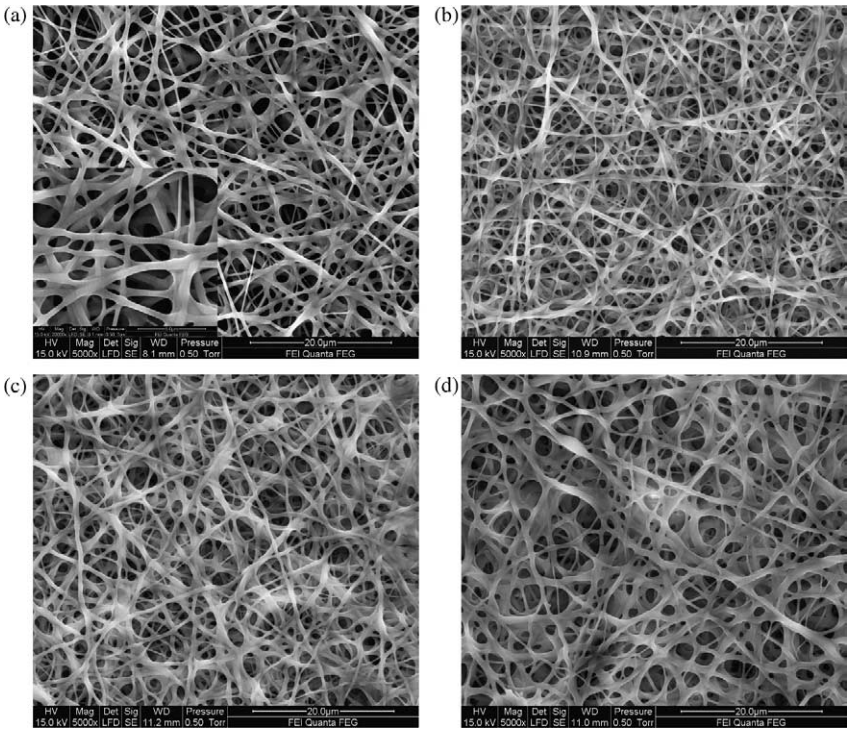


Fig. 2. Crosslinked electrospun gelatin fibers before water-resistant test (a), and immersed in 37 °C DI water for 2 days (b), 4 days (c), and 6 days (d) (samples were subjected to FESEM after drying for 1 week in a vacuum oven).

dimensionally. The colour change is due to the establishment of aldimine linkages (CH=N) between the free amine groups of protein and glutaraldehyde [22,23].

To determine a proper crosslinking condition, grouped gelatin nanofibrous membranes were exposed in the GTA vapor for a timescale of 6, 12 h, 1 day, 2, 3 and 4 days, and then their respective water-resistant behaviors were evaluated and summarized in Table 1. It was found that the samples treated in the GTA vapor up to 6, 12 h and 1 day could be totally dissolved in the 37 °C water after being immersed for 1 day, 2 days, and 3 days, respectively, suggesting an insufficient crosslinking extent. In contrast, the samples crosslinked for more than 2 days seemed able to provide proper crosslinking degree, and, therefore, crosslinking in GTA vapor for 3 days was selected to crosslink gelatin nanofibers.

The morphological, thermal, mechanical and biological properties of the electrospun gelatin fibrous membranes crosslinked under this optimized condition were subsequently characterized. Fig. 2 shows the fiber morphologies of the samples after crosslinking and water resistant tests. Compared with Fig. 1(b), the fibrous form had been grossly preserved, however, due to the nanoscale size of gelatin fibers; the co-existence of water moisture with GTA vapor during crosslinking treatment has affected the fiber morphology to some extent. This is reflected by the fact that fibers at junctions were fused together forming bondings (the inset image of Fig. 2(a)). For the water resistant test in warm water, the fibrous form of gelatin was similarly preserved even after 6 days soaking (Fig. 2(b)–(d)).

### 3.2. Thermal and mechanical properties

Fig. 3 shows the DSC thermograms of raw gelatin powder and the electrospun fibers before and after the crosslinking. Despite gelatin is a denatured substance from collagen involving rupture of the triple-helix structure by breaking of hydrogen bonds and a rearrangement of the triple-helix into a random configuration, under proper conditions, e.g. a gelling process, the chains are able to undergo a conformational disorder–order transition to recover the triple-helix structure [24,25]—a renaturation process. This will be very common for 'solid' gelatin which always contains some water, normally 10–15%, and can be considered as a sol or gel of very high

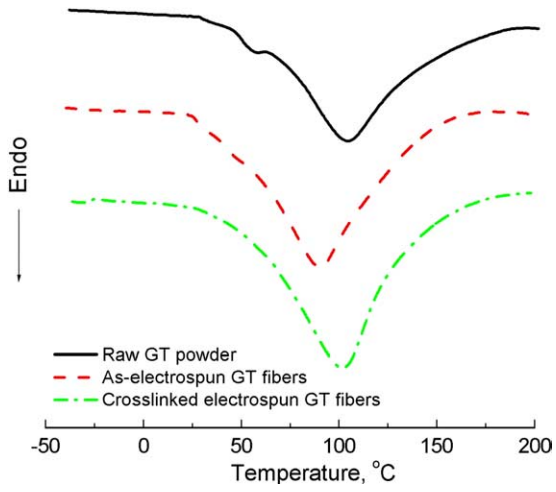


Fig. 3. Typical DSC thermograms of gelatin powder and electrospun fibers.

Table 2
Thermal properties of the electrospun gelatin fibers

| | Raw gelatin powder | As-electrospun gelatin fibers | Crosslinked electrospun gelatin fibers |
|---|---|---|---|
| $T_D$ (°C) | 104.8 | 90.0 | 100.9 |
| $\Delta H_D$ (J/g) | 354.1 | 427.6 | 493.4 |

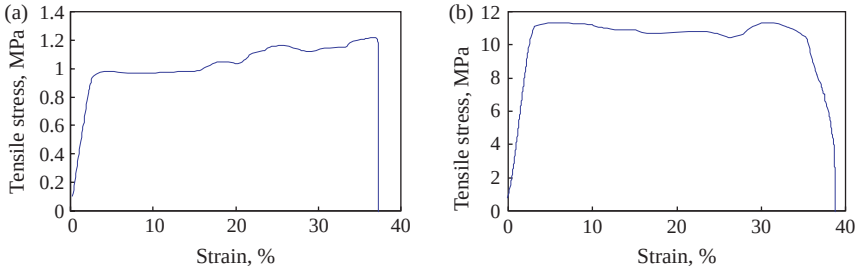


Fig. 4. Typical tensile stress–strain curves of electrospun gelatin fibers before (a) and after (b) crosslinking.

concentration [1]. Therefore, the characteristic endothermic peaks in Fig. 3 have often been termed as denaturation temperature ($T_D$) [26–28], and the corresponding melting heat reflecting the triple-helical content or 'crystallinity' is called denaturation enthalpy ($\Delta H_D$). The values of $T_D$ and $\Delta H_D$, obtained from the raw gelatin powders and the electrospun gelatin nanofibrous membranes before and after the crosslinking were reported in Table 2. It can be seen compared to the raw gelatin material, the $T_D$ of the as-electrospun gelatin fibers was about 15 °C lower but the $\Delta H_D$ was increased. This phenomenon was associated with the electrospinning process [29], which has been explained to result in relatively easy crystallization and increased segmental mobility of the fibrous polymers after this spinning process. The $T_D$ of the crosslinked gelatin membrane was, however, elevated up to nearly 11 °C closing to that of the raw gelatin powder, whereas, the $\Delta H_D$ of the crosslinked membrane was 15 and 40% higher than those of the as-electrospun gelatin fibers and the raw gelatin powder, respectively. The moderate increase in $T_D$ is because the presence of interchain crosslinks within the molecules has little effect on $T_D$. The stability of the helix is primarily dependent on the special features of the peptide bonds involving the imino acids, supplemented by interchain hydrogen bonds at the positions occupied by glycine [30]. It should be noted that all the samples used in the DSC analysis possessed a similar water content of ca. 15.50 wt%, which would exclude the potential influence of water content differences on the denaturation temperature and the enthalpy of helix–coil transition [27], and hence, the differences in their respective thermal behaviors were a consequence of processing and crosslinking. The DSC results indicate the crosslinking treatment has appreciably enhanced the thermal stability of the electrospun gelatin fibers.

Typical tensile stress–strain curves of the electrospun gelatin fibrous membranes before and after the crosslinking were plotted in Fig. 4. The curve shape is similar to that of gelatin films conducted at high relative humidity of 75% [1]. Based on the stress–strain curves, respective tensile properties in terms of tensile strength, Young's modulus, and strain at break are summarized in Table 3. The tensile results indicate the crosslinking treatment dramatically improved the mechanical performance of the gelatin fibrous membrane. After the crosslinking, both the tensile strength and modulus were enhanced to nearly 10 times higher than those of the as-electrospun gelatin membrane. We speculate both the formed inter- and intra-molecular covalent bonds and the bondings between the fiber junctions may be responsible for this tremendous improvement. Formation of point-bonded structures favors the structural integrity of electrospun fibers and hence results in improved mechanical properties [31]. With regard to the elongation, it seems the crosslinking did not reduce the extension ability of the gelatin fibrous membranes. In the contrast, the elongations remained the same or even higher. It is suggested that moisture content could play a greater role than the crosslinking in regulating the elastic and plastic behavior of this natural biomaterial.

### 3.3. Cytotoxicity

Although the crosslinking treatment improved the water-resistant ability and thermal–mechanical properties of the electrospun gelatin nanofibrous membranes, an adverse effect is that such treatment could be cytotoxic to cellular growth during in vitro or in vivo experiments. It has been reported that the potential source of cytotoxicity of the chemically crosslinked biomaterials may be residues of unreacted crosslinking agents and leaching as the materials degrade

Table 3
Tensile properties of the electrospun gelatin nanofibers before and after crosslinking

| | Tensile strength (MPa) | Young's modulus (MPa) | Strain at break (%) |
|---|---|---|---|
| Crosslinked Gt fibers | 12.62±1.28 | 424.7±20.7 | 48.8±5.5 |
| As-electrospun Gt fibers | 1.28±0.12 | 46.5±3.82 | 32.4±7.9 |

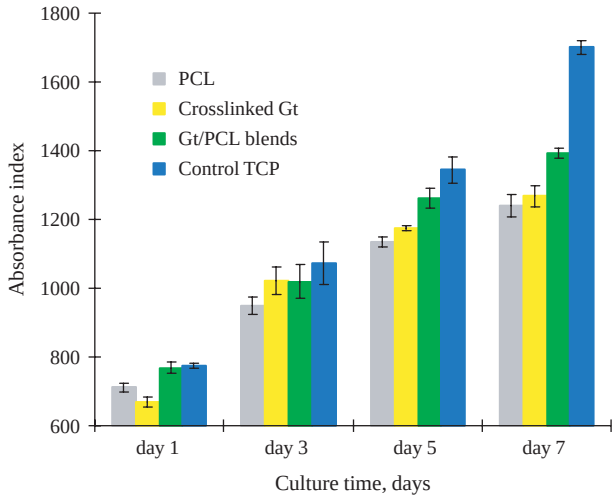


Fig. 5. Comparison of cell proliferation by culturing HDFs on the GTA crosslinked electrospun gelatin fibrous scaffold, and on the controls of TCP substrate, electrospun PCL and Gt/PCL fibrous scaffolds.

[32]. This, however, can be alleviated by thoroughly rinsing the crosslinked material so as to maximally reduce the amount of residual GTA molecules. Fig. 5 gives the cell proliferation results of HDF on the substrates of the crosslinked Gt and controls of TCP, PCL, and Gt/PCL blend assayed at days 1, 3, 5 and 7. The almost linear increases of cell numbers on the crosslinked Gt fibrous scaffolds in a period of 7 days' cell culture indicated that the cell expansion took place on the chemically treated gelatin, as on other control substrates. However, the pronounced lower cell numbers at day 1 culture suggested an initial inhibition of cell proliferation, possibly due to the existence of residual GTA. The slight cytotoxicity could be also evidenced by the fact that the cell expansions on the crosslinked Gt were inferior to those of the Gt/PCL fibrous scaffolds, which can be approximately regarded as the original and water-insoluble form of gelatin fiber. It is consequently suggested that for a better cell proliferation the as-electrospun gelatin fibrous membrane should be crosslinked with crosslinking agents of less cytotoxicity or at least thoroughly rinsed if crosslinked with GTA. Alternatively, we propose electrospinning a complex of gelatin and other biodegradable polymers, e.g. Gt/PCL, would be appropriate in developing gelatin based composite nanofibrous scaffolds for favorable cell–scaffold interactions [33]. Nevertheless, because collageous materials promoting cellular attachement and spreading, the cell proliferation results of the crosslinked gelatin fibrous scaffolds were still slightly superior or comparable with those of the synthetic biodegradable polymer of PCL, which is well known in lacking of bioactivity and cell affinity.

## 4. Conclusions

By exposure of electrospun gelatin nanofibers in a saturated GTA vapor for 3 days, the nanofibrous nonwoven membranes were properly crosslinked. After the crosslinking, the fibrous form was generally preserved even after immersed in 37 °C warm water for 6 days. The crosslinking has also enhanced the thermal stability and mechanical properties. With a combined moisture content of 15.5 wt%, the denaturation temperature increased by ca. 11 °C, whereas, the tensile strength and modulus were improved to nearly 10 times higher than those of the as-electrospun membranes. Cytotoxicity test indicates that the GTA crosslinked fibrous scaffolds could support the proliferation of human dermal fibroblasts. The initial inhibition of cell expansion on the crosslinked gelatin fibrous scaffolds suggested some cytotoxic effect of the residual GTA on the cells. These crosslinked gelatin nanofibers could be suitable for a variety of applications like for tissue engineering scaffolds to improve cell–scaffold interaction, in pharmaceteutical therapy, for medical sutures, as industrial filtration, and so on.

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