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Genipin-Cross-linked Electrospun Collagen Fibers

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Abstract

The fabrication of a fibrous collagen scaffold using electrospinning is desirable for tissue-engineering applications. Previously, electrospun collagen fibers were shown to be unstable in aqueous environments and, therefore, cross-linking is essential to stabilize these fibers. In this study genipin, a significantly less cytotoxic cross-linking agent compared to glutaraldehyde, was used to cross-link electrospun collagen fibers. The significance of this research lies in the use of four alcohol/water solvent systems to carry out the cross-linking reaction to maintain fibrous morphology during cross-linking. The four cross-linking conditions established were: (1) ethanol, 5% water and 3 days, (2) ethanol, 3% water and 5 days, (3) ethanol, 5% water and 5 days, and (4) isopropanol, 5% water and 5 days at a genipin concentration of 0.03 M. Results illustrated that genipin-cross-linking was effective in maintaining collagen fiber integrity in aqueous and cell culture media environments for up to 7 days. In addition, it was shown that fiber swelling could be controlled by using different cross-linking conditions. Swelling of cross-linked fibers immersed in Dulbecco's modified eagle medium for 7 days ranged from 0 to 59 $\pm$ 4%. The cross-linked fibers were analyzed using scanning electron microscopy, Fourier transform infrared spectroscopy and ninhydrin assay. Finally, studies using primary human fibroblasts indicated good cell adhesion to these scaffolds. Overall, our data suggest that these stabilized fibrous collagen scaffolds provide a promising environment for tissue-regeneration applications.

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Keywords
Electrospinning, collagen, genipin, cross-linking, swelling, cell culture, fibrous scaffold, tissue engineering

1. Introduction

Electrospinning is a method of producing long continuous fibers with diameters in the nanometer range. This is done by introducing a polymeric solution into an electric field through a millimeter-diameter nozzle [1]. These fibers find applications in both the industrial and biomedical fields. Of particular interest is the preparation of non-woven fibrous scaffolds for use in tissue engineering of cardiovascular, neural and muscular-skeletal tissues [2–4], since they possess a high surface area to volume ratio, high porosity and excellent pore-interconnectivity. All these characteristics allow for increased cell attachment, enhanced cell migration, and maximized nutrient and waste exchange into and out of the scaffold [5–7].

A wide range of both synthetic and natural biomaterials have been successfully electrospun into fibers [8–12]. For tissue-engineering purposes, type-I collagen is an attractive biomaterial of choice. It is especially desirable as it is an important component of the extracellular matrix that provides a natural environment for a wide range of cells and its stiffness provides mechanical support to the scaffold system. A three-dimensional (3D) organization and alignment of collagen fibers can dictate the final mechanical properties of the scaffold in order to better mimic native tissue environments [13–15]. In addition, cross-linking could increase the tensile strength and toughness of collagen-based scaffolds [16, 17].

Electrospinning, being a relatively simple method for producing fibers, is, thus, attractive for the preparation of type-I collagen fibers for tissue scaffolds. It is also a versatile method as there are many experimental parameters that can be manipulated to control fiber formation, morphology and diameters. Previous attempts to electrospin collagen fibers were shown to be feasible, however the resulting fibers were unstable in aqueous environments [18–22]. Therefore, post-fabrication cross-linking is essential to stabilize the fibers. The most widely used approach is exposing the fibers to glutaraldehyde (GA) vapor for varying lengths of time to achieve the degree of cross-linking required [18–22]. This approach has proven to be rather ineffective since most of the GA cross-linked fibers swell significantly in water and form gel-like structures even after exposure to GA vapor over extended periods of time [19]. GA has also been shown to be highly cytotoxic to cells when released from the cross-linked samples over time [23–26]. This renders GA cross-linked collagen fibrous scaffold highly undesirable. An alternative cross-linking method using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to cross-link type-II collagen has also been reported in the literature [27]. Although the treated fibers exhibited stability when exposed to

an aqueous environment, scanning electron microscopy (SEM) images show a significant degree of swelling and a loss in both the fibrous morphology and porosity [27]. A recent publication compared cross-linking of electrospun fibrinogen fibers using GA, EDC and genipin [28]. In this publication it was shown that both genipin and EDC maintained the mechanical stability of the fibrinogen fibers in PBS for up to 14 days, while fibers cross-linked using GA had no mechanical integrity within the same time period [28]. Also it was shown that EDC cross-linking and genipin cross-linking both maintained a higher porosity compared to the GA-fixed samples [28]. Another recent attempt to cross-link electrospun collagen fibers, using genipin, was carried out in water; the SEM image illustrated significant fiber swelling and fusion between overlapping fibers [29].

Genipin is a natural cross-linking agent that is derived from geniposide found in the fruits of *Gardenia jasminoides* Ellis, which react with primary amine groups [30, 31]. Genipin has previously been utilized for the cross-linking of electrospun natural polymers [28] and has been investigated for the fixation of biological tissues including bovine pericardium and porcine aortic heart valve cusps as a substitute for the highly cytotoxic GA [16, 17, 32–34]. It has also been demonstrated that genipin is approx. 10 000 times less cytotoxic and promotes cell growth approx. 5000 times better than GA [16]. This renders genipin a more attractive cross-linking agent, as compared to GA, for biomedical applications.

We investigated the possibility of using genipin to stabilize electrospun type-I collagen fibers, and we tested the fibers' cell compatibility. Reaction conditions for the control of swelling of the collagen fibers were studied. Degree of cross-linking was determined using the ninhydrin assay. Chemical composition of the cross-linking reaction was studied using Fourier transform infrared (FT-IR) spectroscopy and the *in vitro* cell compatibility was studied using primary human fibroblasts. Results from this study illustrate that the fibrous morphology and 3D structure of the electrospun collagen scaffold are maintained after genipin cross-linking, in aqueous environments of both water and Dulbecco's Modified Eagle Medium (DMEM) for up to 7 days, thus providing an effective approach for the fabrication of collagen-based scaffolds with controlled swelling. In addition, cell cultures of human fibroblasts exhibit cell morphology that more closely parallel that of the natural extracellular matrix environment.

Electrospun collagen fibers are mechanically weak. The as-spun collagen fiber without cross-linking readily disintegrates in an aqueous environment and does not possess any mechanical strength. Those that have been cross-linked and reported in the literature swelled significantly and were not expected to have any mechanical strength either. We report here the preparation of cross-linked electrospun collagen fibers that possess reduced and controlled degree of swelling and, hence, we expect these fibers to possess improved mechanical strength. If we couple this with our ability to produce fibers of controlled orientations, mechanical properties of the collagen fiber scaffold can be further tailored.

2. Materials and Methods

2.1. Materials

All reagents were purchased from Sigma-Aldrich, unless mentioned otherwise.

2.2. Methods

2.2.1. Electrospinning

The electrospinning equipment included a high voltage power supply (Glassman High Voltage) connected to an aluminum plate collector; a syringe pump (Kd Scientific Model KDS 101) placed on a mechanical jack for position control; disposable 1 ml plastic syringes from BD science, and a blunt-ended 18.5-gauge stainless steel needle were used to introduce the collagen solution into the electric field; and an aluminum electrode was attached to the needle to ground it. The electrospinning parameters controlled were voltage (V), tip-to-collector distance (D) and the feed rate (Q). The solution parameter controlled was the collagen concentration. Fibers were collected on aluminum foil that was wrapped around the collector plate.

Rat tail type-I collagen (Sigma-Aldrich) was dissolved at a 5 wt% concentration in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP). The electrospinning parameters that yielded collagen fibers with no beads were: $V = 26$ kV, $D = 13$ cm and $Q = 0.18$ ml/h. All electrospinning was carried out in a fume hood at room temperature.

2.2.2. Genipin Cross-linking

Genipin (purchased from Challenge Bio Products) cross-linking was performed in a solvent system consisting of alcohol and water. Three experimental parameters were varied: (1) the alcohol used (isopropanol *vs* ethanol), (2) water content in solution (0, 1, 3 and 5%) and (3) reaction time (1, 3 and 5 days). The concentration of genipin was fixed at 0.03 M [35, 36].

Cross-linking was performed by placing the collagen fibers in vials containing 20 ml of the cross-linking solution and placing them in a 37°C incubator to speed up the cross-linking reaction. Lids were not screwed tight, to allow exposure to oxygen. After the specified cross-linking period, all samples were stored in isopropanol or ethanol (depending on the cross-linking solvent used during cross-linking) until imaging or cell seeding.

2.2.3. Glutaraldehyde Cross-linking

GA cross-linking was achieved by placing the electrospun samples on top of a 25% GA solution for 24 h. Cross-linking was achieved *via* exposure to GA vapor [4, 12, 13]. The samples were then washed thoroughly in phosphate-buffered saline (PBS) and placed in a 0.1 M glycine solution overnight, to remove any un-reacted GA. A final washing in PBS was carried out to remove excess glycine.

2.2.4. Cell Seeding, Staining and Imaging

Primary human fibroblasts were acquired from the palmar hand fascia of patients that underwent carpal tunnel release surgery. All subjects provided written informed consent under institutional review board approval and specimens were collected with the approval of the University of Western Ontario Research Ethics Board for Health Sciences Research involving Human Subjects (HSREB protocol No. 08222E). This conforms to the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The primary culture obtained from the clinical specimen were maintained in α-MEM + 10% FBS + streptomycin/penicillin until use [37]. All fibroblast cultures were used up to a maximum of 7 passages to ensure an *in vivo*-like morphology and response to their environment.

The cross-linked collagen samples were stored in ethanol, until use, to maintain sterility. Prior to cell seeding the cross-linked samples were first washed thoroughly with PBS, and were then UV sterilized for 30 min (not any longer, to avoid samples from air drying and losing their fibrous structure). Cross-linked collagen samples were then placed in a 24-well plate and seeded with 2.5×10^4 cells/well for 24 h.

After 24 h of cell culture, the samples were fixed using paraformaldehyde for 45 min. Alexa 488 phalloidin and DAPI were used to stain the actin filaments and the cell nuclei, respectively, using standard protocols for fluorescent microscopy. To assess reproducibility, each experiment was repeated three times with different electrospun/cross-linked batches of scaffold.

A cell count was carried out on each sample after 24 h of cell seeding. Five images were taken at 5 different regions (field view of 0.595 mm^2) of each sample ($n = 3$) and the nuclei were counted using Image J (Image processing and analysis in Java, National Institute of Health). This was done to all three runs of each cross-linking condition, for the three different batches (i.e., 45 regions counted per cross-linking condition); the magnification was kept constant for all images.

2.3. Characterization

2.3.1. Image Analysis

A critical point drier EMS-850 (Electron Microscopy Science) with liquid carbon dioxide as solvent was used to dry the samples prior to acquiring SEM images. Samples were dehydrated three times in isopropanol prior to critical point drying.

A Leo 1530 scanning electron microscope was then used to acquire images of both as-spun and cross-linked collagen fibers. The accelerating voltage used was 1 kV, to avoid the use of conductive coating material on the sample surfaces.

Image J was used to measure the fiber diameters. Four SEM images were acquired at different locations for each electrospun sample and 25 fibers were randomly selected and their diameters were measured. Thus, the total fiber diameters measured for each sample were 100 fibers.

Two stages of fiber swelling occur during genipin cross-linking. The first stage is the swelling that takes place due to cross-linking, and the second stage is further swelling that occurs upon exposure to growth media.

To measure the first stage of swelling (due to cross-linking), the diameters of 100 as-spun fibers were measured. The average as-spun fiber diameter was then calculated ($D_{\text{as-spun}}$). The average fiber diameters of cross-linked samples (100 fibers) were also measured ($D_{\text{cross-linked}}$), and the swelling was determined using equation (1):

$$\text{Swelling} = \left(\frac{D_{\text{cross-linked}} - D_{\text{as-spun}}}{D_{\text{as-spun}}} \right) \times 100\%. \quad (1)$$

To measure the second stage of swelling (due to DMEM), each cross-linked sample was cut into two halves. One half was placed in anhydrous ethanol/isopropanol (depending on the solvent used during cross-linking), while the other half was placed in DMEM for the required period of time (2, 5 and 7 days). Comparing two halves of the same sample produced a more accurate degree of swelling. Also, samples that were in DMEM were rinsed for 2 min in distilled water before imaging, to remove any salts that deposited on the fibers with time. Average fiber diameters were then measured after DMEM exposure (D_{final}) and one-way ANOVA using Tukey's test was used to compare the difference between the diameters of cross-linked samples ($D_{\text{cross-linked}}$) and after exposure to growth media for 2, 5 and 7 days (D_{final}). If there was a significant difference, the percent swelling was calculated using equation (2):

$$\text{Swelling} = \left(\frac{D_{\text{final}} - D_{\text{cross-linked}}}{D_{\text{cross-linked}}} \right) \times 100\%. \quad (2)$$

2.3.2. Degree of Cross-linking

A ninhydrin solution was prepared according to Starcher *et al.* [38]. A 4 M sodium acetate buffer was prepared by dissolving 544 g sodium acetate trihydrate in 100 ml glacial acetic acid and 400 ml distilled water. The solution was left to mix overnight and the final pH was measured to be 5.5. A stannous chloride solution was prepared by adding 100 mg $SnCl_2$ to 1 ml ethylene glycol. The resulting solution was cloudy.

The ninhydrin solution was prepared by dissolving 800 mg of ninhydrin in a mixture of 30 ml ethylene glycol and 10 ml 4 M acetate buffer. 1 ml $SnCl_2$ suspension was added and the solution was stirred for 1 h until the final reagent was pale red.

A linear calibration curve was created using different glycine concentrations. Glycine was chosen to calibrate the ninhydrin assay, as mentioned in the literature [17, 36, 39]. Six different concentrations (0, 0.004, 0.0081, 0.0163, 0.0325, 0.065 mg/ml) of glycine were used to obtain the calibration curve. Of the ninhydrin solution 1 ml was added to 2 ml of each of the five glycine concentrations and heated at 80°C for 15 min. The vials were then left to cool for 10 minutes and the absorbance was measured at 570 nm, using a UV-Vis spectrophotometer (Beckman DU series).

Cross-linked samples were dried and weighed (W_{sample}) before performing the assay. Samples were then placed in vials containing 2 ml of distilled water mixed

with 1 ml of the ninhydrin solution. The vials were placed in an 80°C water bath for 15 min and then left to cool down. The cross-linked samples were removed and a Beckman DU spectrophotometer was used to measure the optical absorbance of the solution at 570 nm, which is the typical absorbance for the purple complex that is formed upon the reaction of ninhydrin with amino acids.

After measuring the absorbance, the calibration curve was used to determine the concentration of free amino acids in solution. The mass of free amino acids (W_{free}) was calculated by multiplying the concentration by the volume (3 ml). The ratio of amino acids released from the collagen sample was calculated using equation (3):

$$R = \frac{W_{\text{free}}}{W_{\text{sample}}}. \tag{3}$$

The degree of cross-linking for each combination was calculated using equation (4):

$$R = 1 - \left(\frac{R_{\text{cross-linked}}}{R_{\text{as-spun}}} \right). \tag{4}$$

2.3.3. FT-IR

Infrared measurements were performed using a Bruker Vector 22 Fourier Transform Infrared (FT-IR) spectrometer with an ATR attachment (Pike Technologies). The spectra were collected in absorption mode, using 64 scans, and a resolution of 4 cm^{-1}.

2.4. Statistical Analysis

Statistics were carried out using the OriginPro Version 8.0 (OriginLab Software) statistical software package. Data was analyzed by either a one-way analysis of variance (ANOVA) using a Tukey's test to measure significant differences between multiple groups.

3. Results and Discussion

It is well established that as-spun collagen fibers lose their fibrous morphology upon exposure to an aqueous environment [18–22]. Therefore, cross-linking is essential to both stabilize the fibers and enhance their mechanical properties. It is desirable to use a chemical cross-linking agent with minimum cytotoxicity and be able to maintain controllable fiber morphology. Although GA has been the most frequently used cross-linking agent, many research groups are currently searching for alternatives. Genipin has been proven to be approx. 10 000-times less cytotoxic compared to GA and, therefore, is the subject of this investigation as an alternative cross-linking agent, as the resulting fibers are expected to be more cell-compatible. In addition, it has been demonstrated in the literature that GA-cross-linked collagen fibers swells significantly upon exposure to cell culture media and, thus, the ability to control fiber swelling is essential as it determines the final porosity of the scaffold, pore-interconnectivity as well as mechanical properties of the collagen fibers.

The majority of previous reports has not examined collagen fiber morphology after cross-linking, and we have been unable to find any reports that have presented images after exposure to an aqueous environment for any specific period of time. We systematically investigated the collagen fibers' morphology after cross-linking and after exposure to growth media for up to 7 days. In addition, the degree of swelling in growth media was also determined for up to a week.

3.1. Genipin Cross-linking

The as-spun collagen fibers had an average fiber diameter ± SD of 170 ± 30 nm and contained no beads (Fig. 1). These fibers were stable when dry; however, upon contact with water, they rapidly swelled, and lost their fibrous morphology (Fig. 2). Genipin cross-linking was carried out in an alcohol/water system. Both ethanol and isopropanol were found to be suitable. When absolute ethanol or isopropanol was used as the cross-linking solvent, the cross-linked fibers failed to maintain their fiber morphology at genipin concentrations of 0.03–0.1 M with a cross-linking time of up to 5 days (Fig. 3). However, upon the addition of water to ethanol and isopropanol, certain alcohol/water combinations of the cross-linking solvent system were observed to allow the morphologies of the cross-linked fibers

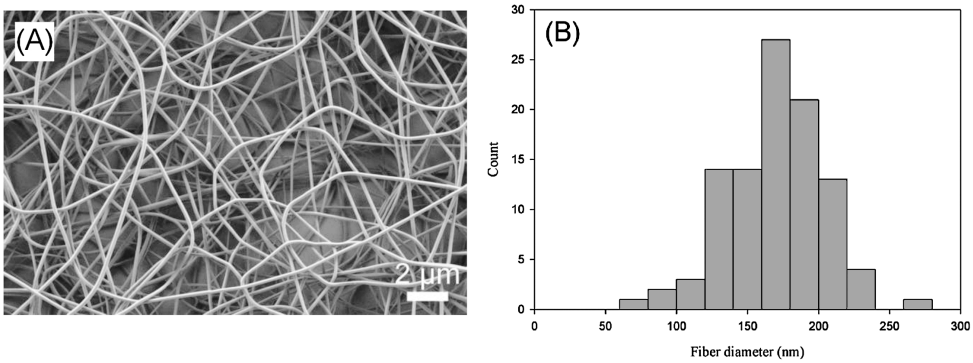

Figure 1. (A) Electrospun collagen fibers and (B) histogram representing the fiber diameter distribution of as-spun collagen fibers with an average fiber diameter of 170 ± 30 nm.

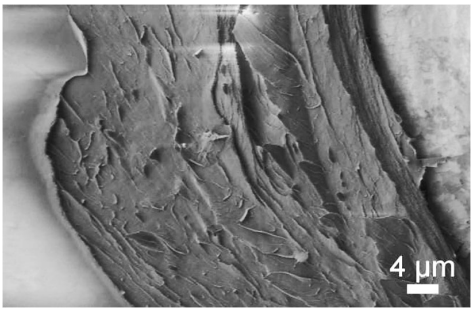

Figure 2. SEM image showing the morphology of electrospun collagen fibers (un-cross-linked) after exposure to distilled water for 5 min.

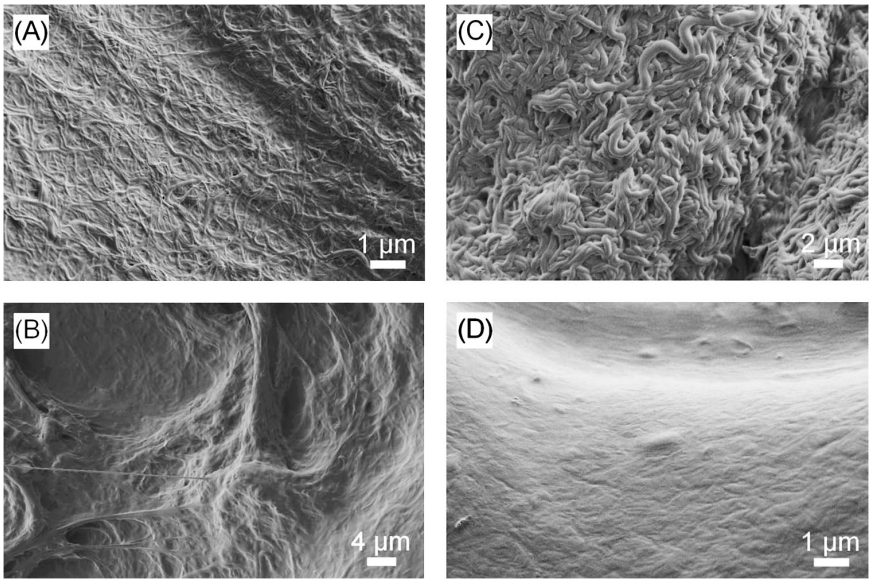

Figure 3. Scanning electron microscope images of collagen fibers after being cross-linked in absolute ethanol solution ((A) before and (B) after exposure to water for 5 min) and absolute isopropanol ((C) before and (D) after exposure to water for 5 min) containing 0.03 M genipin, for 5 days.

to be maintained when exposed to water or DMEM. As a result, a systematic study was carried out to determine the effect of changing cross-linking solution composition and cross-linking time on collagen fiber stability in an aqueous environment. Four cross-linking conditions using three variables (alcohol used, water content and period of cross-linking) that resulted in stable fibers after cross-linking were: (1) ethanol, 5% water and 3 days, (2) ethanol, 3% water and 5 days, (3) ethanol, 5% water and 5 days, and (4) isopropanol, 5% water and 5 days at a genipin concentration of 0.03 M. These cross-linking conditions have been referred to by the assigned numbers throughout the manuscript. Increasing the water content beyond 5% caused significant fiber swelling during cross-linking, and that was the reason for choosing 5% as the maximum limit. In addition, water content below 3% could not maintain the fibrous morphology in an aqueous environment, and that was the reason 3% was set as the lower limit. It was also found that cross-linking for less than 3 days could not maintain the fibrous structure in an aqueous environment.

The genipin cross-linking reaction was associated with a characteristic color change, which could be easily visualized. As the reaction progressed, a greenish color developed initially and eventually became blue [30]. Samples cross-linked using conditions (1) and (3) developed a deep blue color as compared to conditions (2) and (4), which remained green. However, it is important to note that all cross-linked samples turn deep blue after exposure to water; this illustrates the role of water in the blue color formation. Although there have been several studies on the mechanism of the genipin cross-linking, its relationship to the blue color formation is still unknown [30, 40]. Water could have caused swelling in the collagen fibers

which exposed genipin molecules to more amine groups and, thus, formed the deep blue color.

Figure 4 shows SEM images of the genipin cross-linked fibers using the four conditions indicated and after their immersion in DMEM for 7 days. All fibers

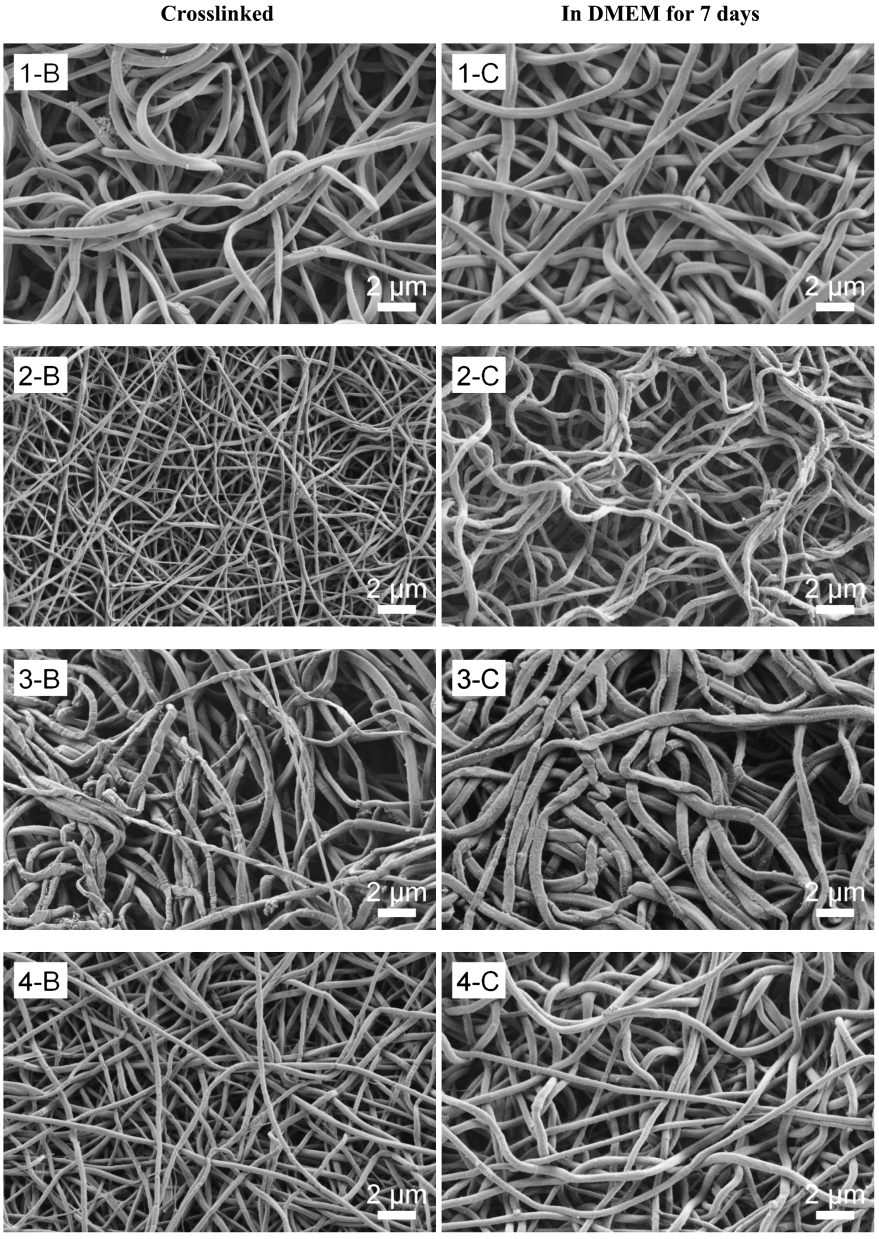

Figure 4. Scanning electron microscope images of cross-linked fibers using the different four conditions (1-B to 4-B), and after placing in Dulbecco's Modified Eagle Medium (DMEM) for 7 days at 37°C (1-C to 4-C).

remained intact for the duration of the test. It was observed that not only the fiber morphology was maintained, but also the degree of swelling among all samples was controllable. These results could be contrasted with those reported based on GA vapor cross-linking. Of the reports on the changes in morphology of the GA cross-linked collagen fibers after exposure to growth media, they showed significant swelling and two reports indicated the formation of a gel-like structure [19, 27]. For applications such as tissue engineering scaffolds, the uncontrolled swelling of the fibers could be problematic since high porosity and pore interconnectivity of the non-woven structure are essential for cell migration and proliferation in the 3D structure.

Figure 5 and Table 1 illustrate the fiber diameters after two stages of swelling; after cross-linking and after exposure to DMEM for 7 days, respectively. There was a significant increase in swelling after cross-linking in all samples ($P < 0.05$). Fibers cross-linked using conditions (1) and (3) (5% water, v/v) had higher fiber swelling after cross-linking compared to condition (2) (3% water, v/v). Condition (4) (5% water, v/v) yielded lower swelling compared to condition (3) most probably due to the higher hydrophobicity of isopropanol compared to ethanol. Cross-linking condition (2) yielded a higher degree of swelling after cross-linking compared to condition (3). This difference is attributed to the differences in water content used between the two conditions; 3 *vs* 5% (v/v), respectively. Thus, it can be concluded that higher water content will lead to more swelling. Also, increasing the time of cross-linking (condition (1) *vs* (3)) did not have a significant effect on fiber diame-

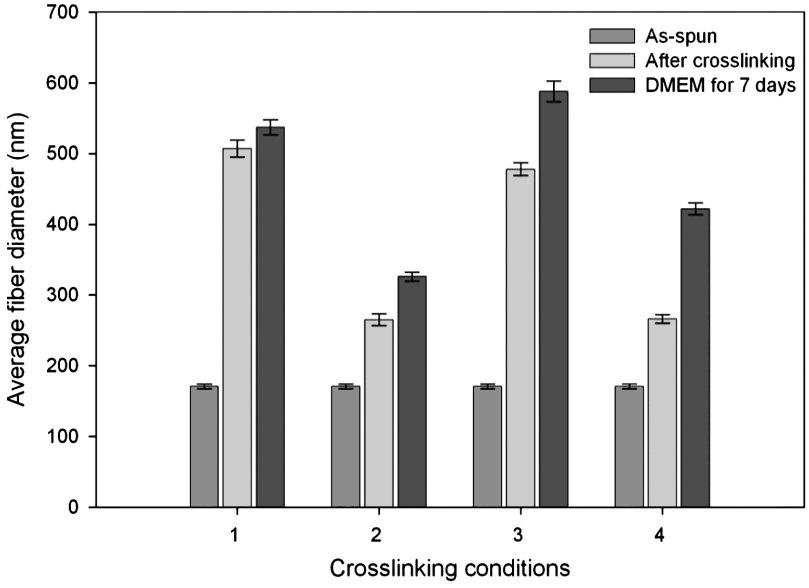

Figure 5. The average fiber diameters of as-spun fibers, cross-linked fibers, and fibers after immersion in Dulbecco's Modified Eagle Medium (DMEM) for 7 days with standard error bars ($n = 100$).

Table 1.
Fiber diameters (D) and percent swelling with standard error (SE) after cross-linking and after exposure to Dulbecco's Modified Eagle Medium (DMEM) for 7 days

Cross-linking conditions	Swelling post-cross-linking			Swelling post-immersion in DMEM for 7 days		
	$D_{\text{as-spun}} \pm$ SE (nm)	$D_{\text{cross-linked}} \pm$ SE (nm)	Swelling (%)	$D_{\text{cross-linked}} \pm$ SE (nm)	$D_{\text{DMEM}} \pm$ SE (nm)	Swelling (%)
1	171 ± 3	510 ± 10	196 ± 8	510 ± 10	540 ± 10	0
2	171 ± 3	265 ± 8	55 ± 5	265 ± 8	326 ± 6	24 ± 3
3	171 ± 3	478 ± 9	180 ± 7	478 ± 9	590 ± 20	23 ± 4
4	171 ± 3	266 ± 6	56 ± 4	266 ± 6	422 ± 8	59 ± 4

ters after cross-linking ($P > 0.05$). This illustrates that the water content has a more significant effect on fiber morphology compared to cross-linking time (3 *vs* 5 days).

Fibers cross-linked using condition (1) did not have any statistically significant swelling for up to 7 days immersion in DMEM (Table 1). The increase in swelling in relation to cross-linking time (condition (3) *vs* (1)) could be attributed to the oligomerisation of genipin with time, which may have increased porosity of the fibers and allowed more swelling to take place [41, 42]. Cross-linking conditions (2) and (3) yielded similar degrees of swelling after exposure to DMEM for 7 days, which suggests that the cross-linking reaction is driven to completion after 5 days. Cross-linking condition (4) yielded fibers with the highest degree of swelling after exposure to DMEM, and this could be attributed to the hydrophobic effect of isopropanol (compared to ethanol), which impeded the genipin molecules from cross-linking between two primary amine groups, and thus allowed for greater swelling. A more thorough explanation of why this might be the case is presented in the FT-IR analysis (see below) of fibers cross-linked using condition (4). Overall, these results illustrate the control of degrees of swelling, in DMEM, that are achievable by the judicious choice of cross-linking conditions.

3.2. Degree of Cross-linking

The degree of cross-linking of collagen fibers can be measured using the ninhydrin assay [16, 38, 43]. This assay detects the amount of free amino acids in solution by forming a purple complex upon the reaction of ninhydrin with primary amine groups. Thus, the more cross-linked the collagen sample, the less free primary amine groups are available for the ninhydrin reaction and the lower is the purple color intensity determined at a wavelength of 570 nm. However, the ninhydrin assay cannot be used to determine the density or nature of cross-linking, since two genipin molecules that react with one primary amine group each and do not cross-link with another, will give the same result as a genipin molecule cross-linking two primary amine groups [44].

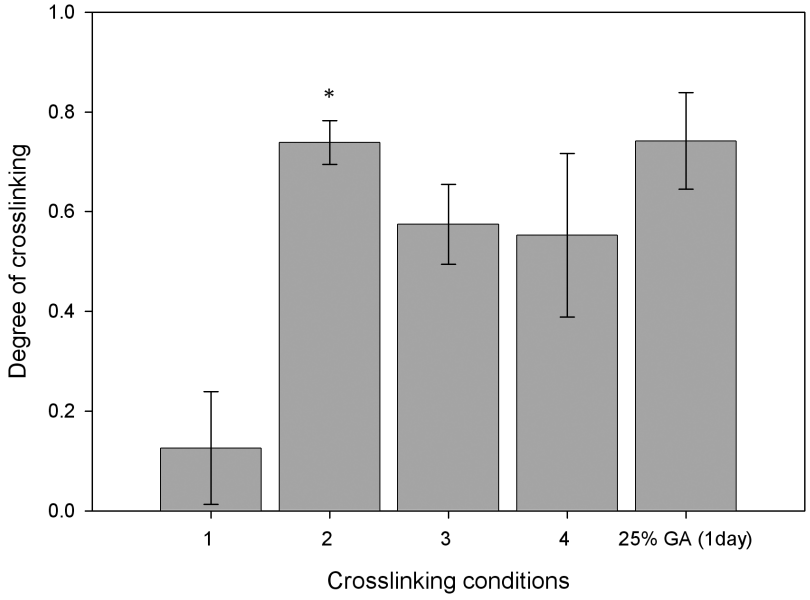

Figure 6. The degrees of cross-linking with standard error bars, measured using the ninhydrin assay ($n = 3$).

Figure 6 illustrates the degrees of cross-linking for all cross-linking conditions as determined by the ninhydrin assay. A GA cross-linked sample is included for comparison. It can be seen that all cross-linking conditions are effective to varying degrees. Samples cross-linked using condition (3) had less free primary amine groups compared to condition (1) (i.e., higher degree of cross-linking). Condition (2) yielded the highest degree of cross-linking ($P < 0.05$). It is hypothesized that due to the lower water content used in condition (2) (3%, v/v) compared to conditions (1) and (3) (5%, v/v), less swelling took place in the fibers during cross-linking (confirmed by the results shown in Fig. 5) and thus most of the cross-linking took place on the surface of the fibers. The increased cross-linking density at the surface could have hindered the ninhydrin from accessing with free amine groups in the core of the fibers, which resulted in an apparent higher degree of cross-linking. This correlates well with the lower degree of fiber swelling post exposure to DMEM, due to the inability of water to infiltrate the cross-linked fibers. Cross-linking condition (4) was comparable to condition (3); however, the differences in the nature of cross-linking cannot be determined by the ninhydrin assay. GA cross-linked samples yielded a high degree of cross-linking within 1 day, which illustrates that the GA cross-linking reaction proceeded at a faster rate than its genipin counterpart [44].

3.3. FT-IR Characterization

FT-IR was used to delineate the chemical nature of the genipin cross-linking reaction. The reaction between primary amine groups in the as-spun collagen fibers and genipin is evident from the disappearance of a peak at 1133 cm^{-1} from the as-spun

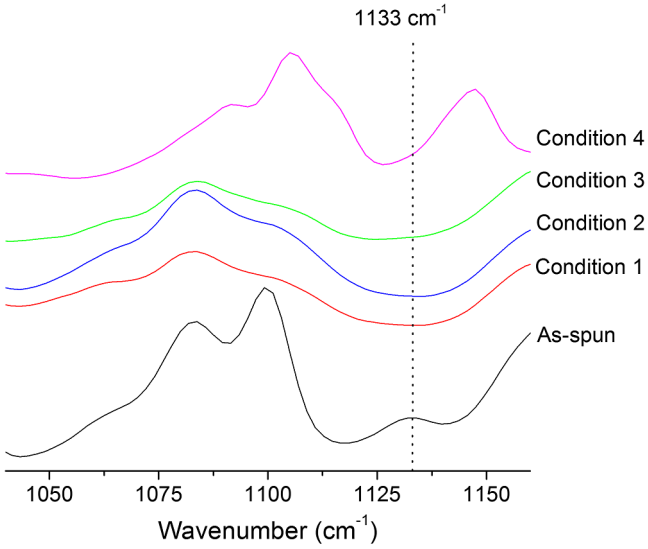

Figure 7. FT-IR spectra (1050–1150 cm^{-1}) of as-spun collagen fibers and fibers cross-linked using the four conditions; it is observed that the C–N stretch vibration of the primary aliphatic amine (–CH–NH_2) at the absorption at 1133 cm^{-1} is present in the as-spun fibers; however, it is absent in all the cross-linked fibers, indicating cross-linking has occurred. This figure is published in colour in the online edition of this journal, which can be accessed *via* http://www.brill.nl/jbs

collagen fibers spectrum upon cross-linking (Fig. 7). This peak is attributed to the C–N stretching vibration of a primary aliphatic amine, which is available in lysine and arginine [45]. As seen in Fig. 7, this peak disappears in all the cross-linked samples, and thus is taken as an indication of the genipin-primary amine group cross-linking reaction. A secondary amide is also formed after genipin cross-linking of collagen and has a C=O stretching vibration in the range 1680–1630 cm^{-1}. However, since collagen's amide I and II peaks are also in this range, it is not possible to quantify the increase in secondary amide groups after genipin cross-linking [46, 47].

The FT-IR spectra of collagen fibers cross-linked using conditions (1), (2) and (3) are very similar, which indicates similar chemical reactions and cross-linked products. However, cross-linking using condition (4) in aqueous isopropanol yielded a FT-IR spectrum which is not quite the same as compared to the other three conditions. From Fig. 8 it can be seen that the cross-linked collagen fibers show a spectrum that is more similar to that of genipin, indicating that the cross-linking reaction in the less hydrophilic isopropanol solution may be quite different from that in the aqueous ethanol solution. Butler *et al.* proposed two cross-linking reactions that take place between genipin molecules and primary amine groups [30]. The first reaction is a nucleophilic attack of the primary amine group on the C3 carbon of genipin to form an aldehyde group. The secondary amine group then attacks the aldehyde group and results in a tertiary amine group, thus cross-linking the primary

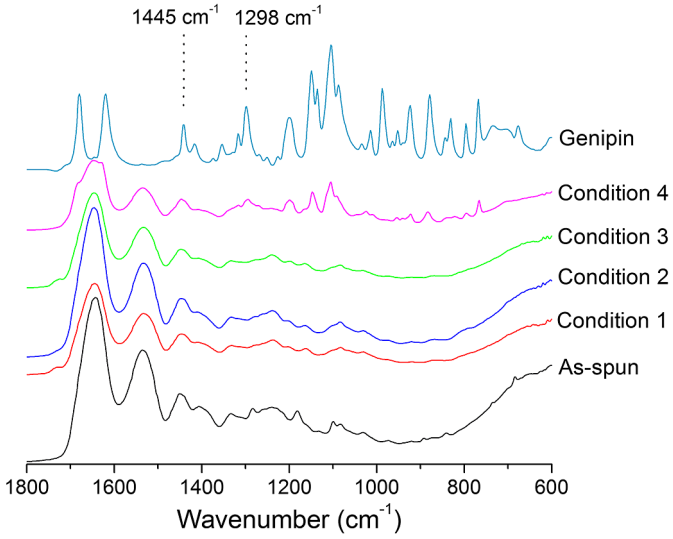

Figure 8. FT-IR spectra of genipin, as-spun collagen and the genipin-cross-linked collagen fibers using the four cross-linking conditions. This figure is published in colour in the online edition of this journal, which can be accessed *via* http://www.brill.nl/jbs

amine groups and genipin. The second slower reaction is a S_N2 nucleophilic substitution that involves the replacement of the ester group on genipin by a secondary amide linkage [30]. Interestingly, it can be observed that the two peaks, 1298 and 1445 cm^{-1}, that represent the C–O–C asymmetric stretch and the CH_3 bend of the methyl ester, respectively, are present in collagen samples cross-linked using condition (4), implying that the ester groups in genipin are not fully reacting to form cross-links. Therefore, it can be interpreted that the second cross-linking reaction, which takes place at the ester group, is slowed down in an isopropanol-containing reaction medium.

3.4. Cell-Culture Studies

3.4.1. Cell Attachment Studies

Primary human fibroblasts cultures were used to assess cell compatibility of the genipin cross-linked collagen fibers. Cell morphology using collagen fiber mats prepared using the four cross-linking conditions as substrates compared to uncross-linked collagen fibers are summarized in Fig. 9. Figure 9 shows the filamentous (F-)actin cytoskeleton and nucleus of these cells stained with phalloidin and DAPI, respectively. These fluorescence microscopy images demonstrated that primary human fibroblasts attached well to both the un-cross-linked and genipin cross-linked collagen samples. The as-spun collagen fiber sample shown in Fig. 9A can be considered as a gel upon exposure to cell culture media, since the fibers swell and disintegrate rapidly to form a continuous layer (see Fig. 2). Therefore, the fibroblast morphology on un-cross-linked collagen fiber substrate is similar to those reported for a collagen gel in the literature [48]. The cells acquired a flattened morphology,

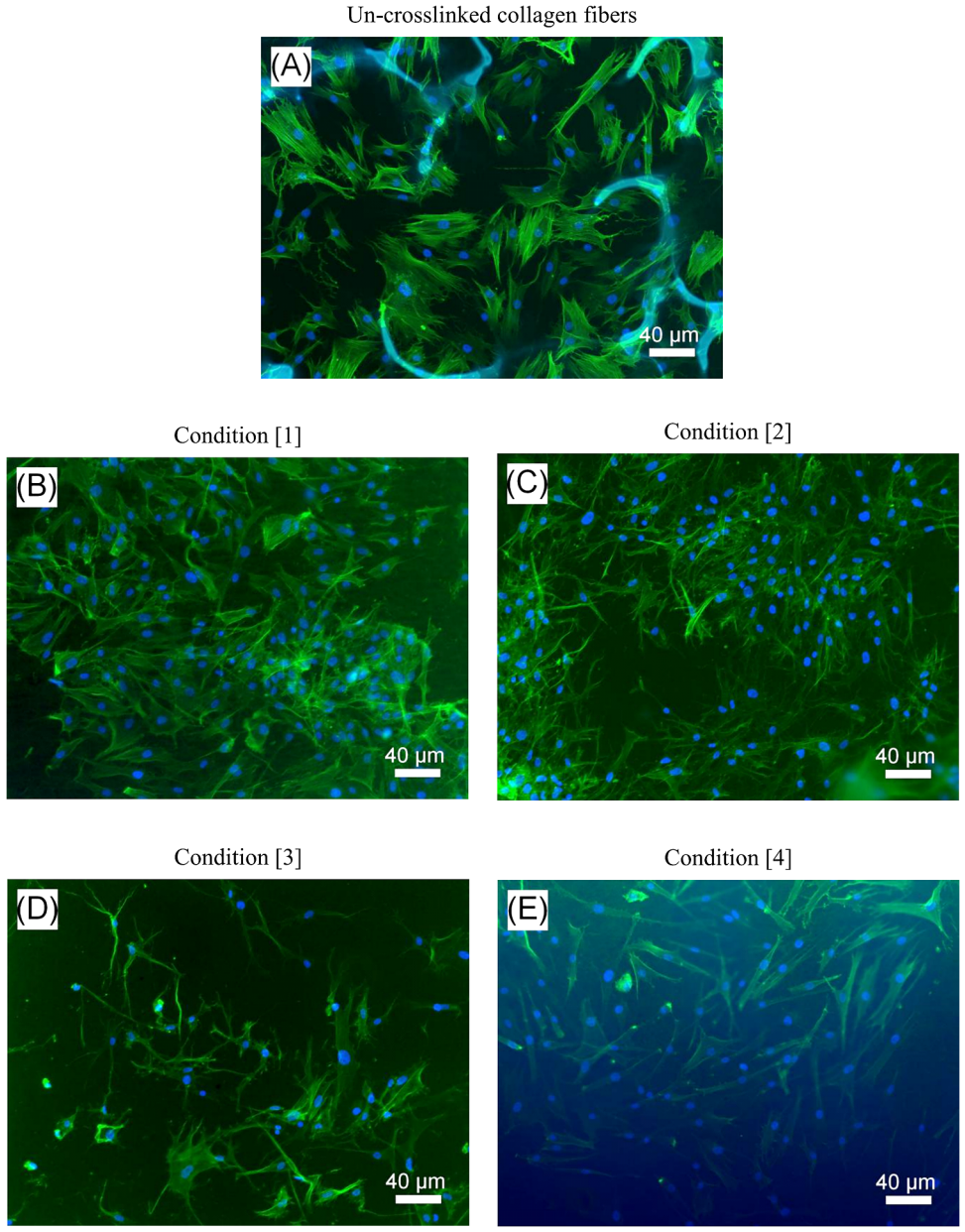

Figure 9. Fluorescence microscopy images of primary human fibroblasts cultured on a collagen gel (2D) and genipin-cross-linked collagen fibrous scaffolds using the four conditions. This figure is published in colour in the online edition of this journal, which can be accessed *via* http://www.brill.nl/jbs

with the appearance of stress-aggregated stress fibers and few extensions, potentially due to the isometric tension that was experienced by the cells [49].

On the other hand, fibroblasts seeded on the genipin cross-linked collagen fiber scaffolds acquired bipolar/stellate morphologies (Fig. 9B–E) which are similar to

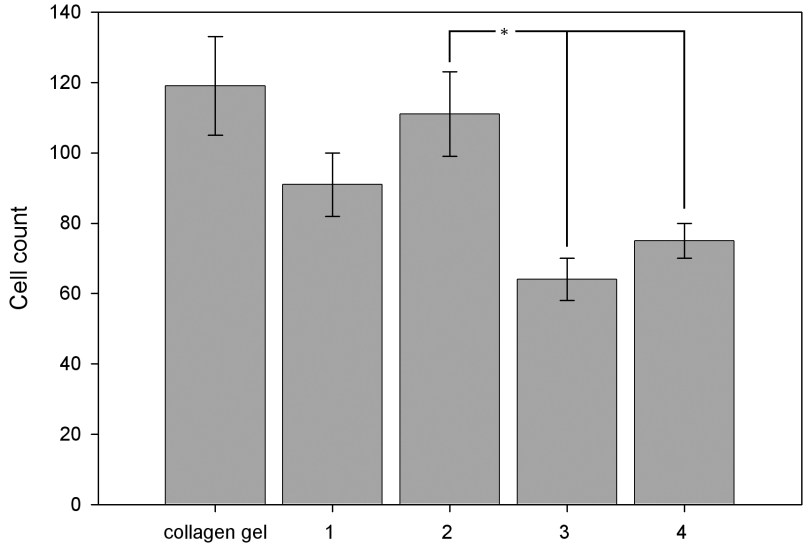

Figure 10. Cell counts carried out after 24 h of cell-seeding on un-cross-linked collagen gels, and samples cross-linked using the four conditions ($n = 45$).

fibroblasts seeded on stressed 3D constructs reported in the literature [48, 49]. Since the collagen fibers are randomly distributed and are cross-linked to varying degrees, fibroblasts adhering to these fibers are likely to experience varying degrees of surface tension. Cell morphologies observed were more akin to *in vivo* morphologies and may provide a more favorable environment for tissue regeneration. Figure 10 shows the cell count on the collagen gels and all the genipin-cross-linked samples. There was no statistical difference between the cell count on collagen gel and cross-linked samples ($P > 0.05$). However, there was a significant difference between the samples cross-linked using condition (2), compared to conditions (3) and (4) ($P < 0.05$). The difference in cell counts amongst cross-linked samples is a preliminary indication of the cross-linking conditions that are more cell-friendly. In order to confirm this, future work will be required to study cellular proliferation (up to 7 days) and migration into the scaffold.

4. Conclusion

Type-I collagen fibers were successfully fabricated by electrospinning and cross-linked using genipin. Four cross-linking conditions were developed to cross-link the fibers, while maintaining their fibrous morphology. The degrees of swelling and cross-linking could be varied by choosing from the different cross-linking conditions, depending on the porosity and degradation rate required for different tissue engineering applications. FT-IR analysis gave insight into the genipin cross-linking process. Primary human fibroblasts attached to all the cross-linked samples after 24 h of culture. Overall, this manuscript illustrates the success of the first optimiza-

tion step, which is to maintain the fibrous morphology in an aqueous environment, and to illustrate that cell attachment was successful after 24 h of culture. Ongoing work involves further assessing cellular proliferation on the cross-linked samples for up to 7 days. In addition, the mechanical and thermal properties will be assessed using Atomic Force Microscopy (AFM) and Differential Scanning Calorimetry (DSC), respectively.

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