Storage stability of electrospun pure gelatin stabilized with EDC/Sulfo-NHS

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ABSTRACT

With the rapid development of biomimetic polymers for cell-based assays and tissue engineering, crosslinking electrospun nanofibrous biopolymer constructs is of great importance for achieving sustainable and efficient 3D scaffold constructs. Uncrosslinked electrospun gelatin nanofibrous constructs immediately and completely dissolved in aqueous solutions due to their aqueous solubility and poor storage stability. Here, a novel and versatile approach for the fabrication and crosslinking of electrospun gelatin construct with tunable porosity and high aspect ratio nanofibers is presented. Uncrosslinked electrospun gelatin/genipin nanofibrous and pure gelatin nanofibrous constructs exhibited smooth surfaces that were well-defined, with a diameter in the range of 448 ± 364 nm and 257 ± 57 nm, respectively. Dehydrothermal, genipin-EDC/Sulfo-NHS, and EDC/Sulfo-NHS crosslinking approaches were examined to achieve insoluble gelatin nanofibrous constructs that were suitable for cell-based assays. Mechanical characterization demonstrated that the pure gelatin nanofibrous construct crosslinked via EDC/Sulfo-NHS exhibited an increased mechanical strength and stiffness and showed no dissolution in aqueous solutions and retained its fiber morphology. An excellent one month storage stability was demonstrated at 22, 4, -20, and -80 °C (dehydrated) and at 4 °C (hydrated). The as-crosslinked gelatin nanofibrous construct was highly biocompatible (90% cell viability), as demonstrated by the promoted proliferation of PC12 cells.

Keywords: Gelatin, genipin, EDC/S-NHS, crosslinking, storage stability

Introduction

Electrostatic spinning (electrospinning) of bio-mimicking nanofibers has emerged as a useful platform for various advanced applications in the biomedical field for fabricating nanofiber constructs for drug delivery [1], wound dressing [2], and more recently for three-dimensional (3D) scaffolds for tissue engineering [3-6]. Various synthetic and natural polymeric nanofibers and blend of biocompatible polymers have been investigated as supporting fibrous structures [7-10]. In addition, electrospinning has become the most commonly used technique in the preparation of nanofibrous scaffolds because of the high surface area to volume ratio and inherent high porosity and interconnected porous network exhibited in the nanofiber constructs when compared to nanofibers fabricated using solvent casting and phase-separation techniques [11]. Since cells are naturally endowed with nanofibers in the cytoskeleton and the native extracellular matrix (ECM), electrospun nanofibers have a major role to play in defining the mechanical properties and various important cellular functions in biological systems. These electrospun nanofibers can provide various mechanisms to restore functions or achieve favorable responses desired in nanofiber construct models for tissue engineering applications [12].

Despite its biomimetic potential and advantages to provide mechanistic strategies for cell adhesion, proliferation and viability, nanofibrous scaffold constructs continue to be plagued by their very poor mechanical properties, fast degradation rate, and complete dissolution upon immersion in aqueous solutions. Crosslinking has the ability to enhance the mechanical and chemical solubility of the electrospun nanofibers. The most common chemical crosslinking techniques employ glutaraldehyde (GA), 1-ethyl3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), genipin and enzymatic crosslinking, whereas the most common physical crosslinking techniques employ dehydrothermal (DHT), plasma, and ultraviolet (UV) treatments [13]. Generally physical crosslinking of the nanofibers results in a low crosslinking density, whereas chemical crosslinking results in higher crosslinking densities [13,14]. The disadvantage of employing chemical crosslinking is that it can result in structural and chemical changes in the nanofibrous constructs and can take anywhere from 24*h* to 5 days to complete. Although genipin crosslinking of nanofiber construct results in highly cross-linked nanofibers, the nanofibrous construct changes from a relatively transparent white construct to an opaque dark blue nanofibrous construct depending on the crosslinker concentration and duration of crosslinking. On the other hand, crosslinking approaches employing glutaraldehyde to crosslink electrospun fibers are short lived in aqueous solution. These commonly used crosslinking techniques can be cytotoxic to cells upon the release of unreacted residues during the degradation of the nanofibrous construct and thereby, render genipin and glutaraldehyde based electrospun nanofibers unfavorable for 3D cultivation of cells in tissue engineering. While the combination of EDC and N-hydroxysuccinimide (NHS) crosslinking strategies for nanofiber constructs have been widely employed for gelatin based biopolymers [15], Genipin/EDC/N-hydroxysulfosuccinimide (Sulfo-NHS) has not been used. To our knowledge, this is the first report that shows the effect of 7*h* crosslinking with EDC/Sulfo-NHS on the storage stability of crosslinked gelatin nanofibrous construct that was performed on dehydrated and hydrated platforms. The EDC/Sulfo-NHS crosslinked gelatin nanofibrous construct was evaluated in terms of their morphology, mechanical strength, storage stability and biocompatibility.

Although collagen is the most abundant protein in the extracellular matrix (ECM) [16-18], exogenous collagen has been shown to induce antigenic and immunogenic response in vivo due to its helical structure and amino acid sequences [19]. Since gelatin is derived from partial hydrolysis of natural collagen, it lacks both tyrosine and tryptophan and exhibit very low levels of phenylalanine amino acids [15]. This makes gelatin less likely to form aromatic radicals, thereby reducing the potential of antigenic response in vivo [20]. The lower immunogenicity and availability of gelatin at relatively low cost make gelatin an excellent biodegradable material for applications in pharmaceutical, medical applications, and 3D scaffold preparation [15,19-20]. However, electrospun gelatin is readily soluble in aqueous systems, and can be readily digested by collagenase, an enzyme secreted by a variety of cell lines. As a result, its application in cell-based assays and tissue engineering is limited. More emphasis must be placed on retaining the original fiber morphology of electrospun gelatin scaffolds upon immersion in aqueous environments.

Materials and Methods

Fabrication of the Electrospun Nanofibers: Two separate gelatin solutions were prepared by dissolving gelatin from Bovine Skin Type B Powder (Sigma-Aldrich) in 70/30 vol.% acetic acid/double distilled water at a concentration of 30% (w/v). The second gelatin solution was prepared with the addition of genipin 3% (w/w) in a solution of ethanol and 1X PBS (1:2 volume ratio). All solutions were kept under magnetic stirring at 50 °C for 1h before electrospinning. The prepared gelatin solutions were electrospun into nanofibrous constructs using the following conditions: an applied voltage of 15 kV was maintained between the needle tip and the collector, the needle to collector distance was maintained at 15 cm, and the solution flow rate was maintained at 5 µl/min. The electrospinning was achieved at room temperature and a relative humidity of less than 40%. The electrospun constructs were kept at room temperature overnight in order to remove the residual solvents and these samples were used for further characterizations.

Physical and Chemical Crosslinking of Electrospun Nanofibers: The as-spun gelatin/genipin nanofibrous construct was cut into 2.5 cm x 2.5 cm pieces. Each piece was fixed into plastic crowns (CellCrown™, Scaffdex) prior

to crosslinking. The fixed dry nanofibrous constructs were placed in 12-well tissue culture plates. The samples were crosslinked via different concentrations of EDC (Sigma-Aldrich)/Sulfo-NHS (Fisher Scientific) prepared with 90% ethanol for 7h at room temperature (22 °C) and relative humidity of 40% under moderate agitation. Samples were treated with various molar ratios of EDC/Sulfo-NHS (2:1, 2.48:1, and 2.84:1). Approximate 2.5 ml of EDC/Sulfo-NHS crosslinking solution was poured onto the fixed gelatin/genipin nanofibrous constructs. Additional samples were crosslinked via dehydrothermal treatment (DHT) at 160 °C for 48h in a vacuum oven (22 mm Hg). In addition, nanofibrous construct electrospun from pure gelatin was crosslinked via 2:1 molar ratio EDC/Sulfo-NHS. Upon chemical crosslinking, the samples were washed 5 times with 1X phosphate buffer saline (PBS) at room temperature. Each time, the samples were immersed in fresh 1X PBS for 30 min under mild agitation. Subsequently, the as-spun gelatin/genipin crosslinked (2:1 molar ratio of EDC/Sulfo-NHS) samples were stored in either 1X PBS or cell culture medium (DMEM–Dulbecco's Modified Eagle Medium) for 5h and 5 days. An extended storage stability characterization was performed with these as-spun pure gelatin samples stored at -80, -20, 4, and 22 °C in the dehydrated state and at 4, 22, and 37 °C in 1X PBS (hydrated state) in order to investigate the degradation of the crosslinked gelatin nanofibrous construct after 14 and 30 days of storage (triplicate samples were used for statistical purposes).

Morphological Characterizations: The surface morphology and fiber diameter of the as-spun nanofibrous constructs were observed using high resolution scanning electron microscopy (FEI Nova NanoSEM 450) operating at an acceleration voltage of 20 kV. All samples were sputter coated with ~6 nm gold using a sputter coater (MNT-JS1600 Plasma Sputtering Coater, MicroNanoTools) and were mounted using carbon tape on aluminum SEM stubs. Approximately 50 individual nanofiber diameters were acquired from the SEM micrographs using an image analysis software (Image J, National Institutes of Health, USA). A two-point measuring analysis was used to calculate the average nanofiber diameter and standard deviation.

Thermogravimetric analysis (TGA): Thermal degradation behavior of the as-spun nanofibrous constructs were analyzed by Thermogravimetric Analyzer ((Pyris 1 TGA, PerkinElmer, USA) with a heating rate of 20 ℃/min from 50 ℃ to 800 ℃ and holding at 800 ℃ for 5 minutes. Thermogram was recorded using Pyris software.

Fourier transform infrared (FTIR) spectroscopy: Fourier transform infrared spectra (FTIR) were obtained (Spectrum Two FT-IR Spectrometer, PerkinElmer, USA) with attenuated total reflectance accessory (ATR) using diamond crystal. For each spectrum applied force was 100 N and 5 scans were accumulated at 4 cm^{-1} resolution in the range of 4000-450 cm^{-1}.

Mechanical Testing: The mechanical properties of the as-spun gelatin nanofibrous constructs were measured with a ElectroPuls E1000 Testing System (Instron, Norwood, MA). Tensile properties of the uncrosslinked and crosslinked constructs were measured. The gelatin nanofibrous constructs were cut into rectangular shape with a width of 1 cm and an effective length of 5 cm. The thickness of the uncrosslinked and crosslinked gelatin nanofibrous constructs were on approximately 60-80 nm and 30-40 nm, respectively. Each piece was loaded into a load cell, wherein the sample was stretched along the 5 cm side at a test rate of 0.1 mm/sec. The resulting stress-strain curves were collected at a data acquisition rate of 120 Hz and used to calculate the elastic modulus and tensile strength. Measurements were performed on five samples per material. All of the stress-strain curves were recorded at 22 °C and 20% relative humidity.

Cell Culture: PC-12 cells (ATCC CRL-1721) were cultured in DMEM (Dulbecco's Modified Eagle Medium, Thermo Fisher Scientific) supplemented with 10% calf serum in an incubator at 37 °C with 5% CO_2. Nanofibrous constructs were fixed in plastic crowns and then disinfected with 70% ethanol followed by UV irradiation for 30 min. Subsequently, the UV sterilized nanofibrous constructs were rinsed three times with 1X PBS before seeding with PC-12 cells. A cell density of 2 x 10^6 cells ml^{-1} was seeded onto the surface of gelatin nanofibrous construct and was incubated at 37 °C for 20 min to allow the cells to adhere. Same number of cells was seeded in the wells of tissue culture plate which served as control. Afterwards, 1.5 ml fresh media was gently added to the well containing the crown insert without disrupting the cells on the nanofibrous construct. Adherent PC-12 cells cultured for 2 days were visualized and analyzed by two molecular probes, calcein-AM (Invitrogen) and ethidium homodimer-1 (EthD-1) (Invitrogen) in order to determine cytocompatibility of the crosslinked gelatin nanofibrous construct in comparison to tissue culture polystyrene. Stained PC-12 cells were imaged by confocal microscopy (Leica SP5, Leica Microsystems, Germany). PC-12 cells adhesion on the crosslinked gelatin nanofibrous construct was fixed on with 4% formaldehyde

and then permeabilized by immersion in 0.2% Triton for 5 min. Afterwards, the nuclei was stained with 4′,6-diamino-2′-phenylindole (DAPI) (Thermo Fisher Scientific). Cell viability and adhesion were calculated from cell counts of images obtained through raster patterning (5 images per specimen) of 3 specimens.

Results and Discussion

Morphology of as spun nanofibrous constructs: Crosslinking strategies have been developed to improve the insolubility and mechanical stability of electrospun gelatin nanofibers [15]. Fig. 1A – D shows the SEM micrographs of electrospun gelatin/genipin nanofibrous construct crosslinked via dehydrothermal treatment (DHT). Although this physical crosslinking method does not introduce cytotoxic residues into the crosslinked system, it is apparent that the degree of crosslinking achieved by this method is relatively low (Fig. 1 A-B), wherein the nanofibers morphology remained relatively the same with a smooth surface and uniform diameters along their lengths. The diameter of the uncrosslinked gelatin/genipin nanofibers was observed to be approximately 448 ± 364 nm, whereas the DHT crosslinked nanofibers exhibited diameters of about 640 ± 321 nm. This represents a 42.64% increase in the nanofibers diameter upon formation of inter-chain amide bonds through condensation [1,2], thereby enhancing the sturdiness of the crosslinked gelatin nanofibrous construct in comparison to uncrosslinked gelatin nanofibrous construct [21,22]. However, the similarity between the uncrosslinked and DHT crosslinked micrographs indicates that this strategy results in nanofiber construct with some solubility in aqueous environments, where the nanofibers were observed to fuse together and flatten after exposure to 1X PBS (Fig. 1 C) and cell culture media (Fig. 1D) to the point where the fiber morphology is indiscernible. It is apparent that the DHT crosslinked gelatin nanofibrous construct undergoes partial dissolution in 1X PBS and cell culture media; however, uncrosslinked gelatin nanofibrous construct was observed to completely dissolves in both aqueous environments. As a result, the gelatin/genipin nanofibrous construct crosslinked via DHT treatment is not

suitable for 3D cell-based assays or tissue engineering due to its solubility and poor fiber morphology retention in PBS and cell culture media.

Both non-zero and zero length chemical crosslinking strategies involving EDC/Sulfo-NHS were employed to crosslink gelatin/genipin nanofibrous construct. The morphology and fiber diameter of the construct before and after crosslinking in various molar ratios of EDC/Sulfo-NHS were investigated. As shown in

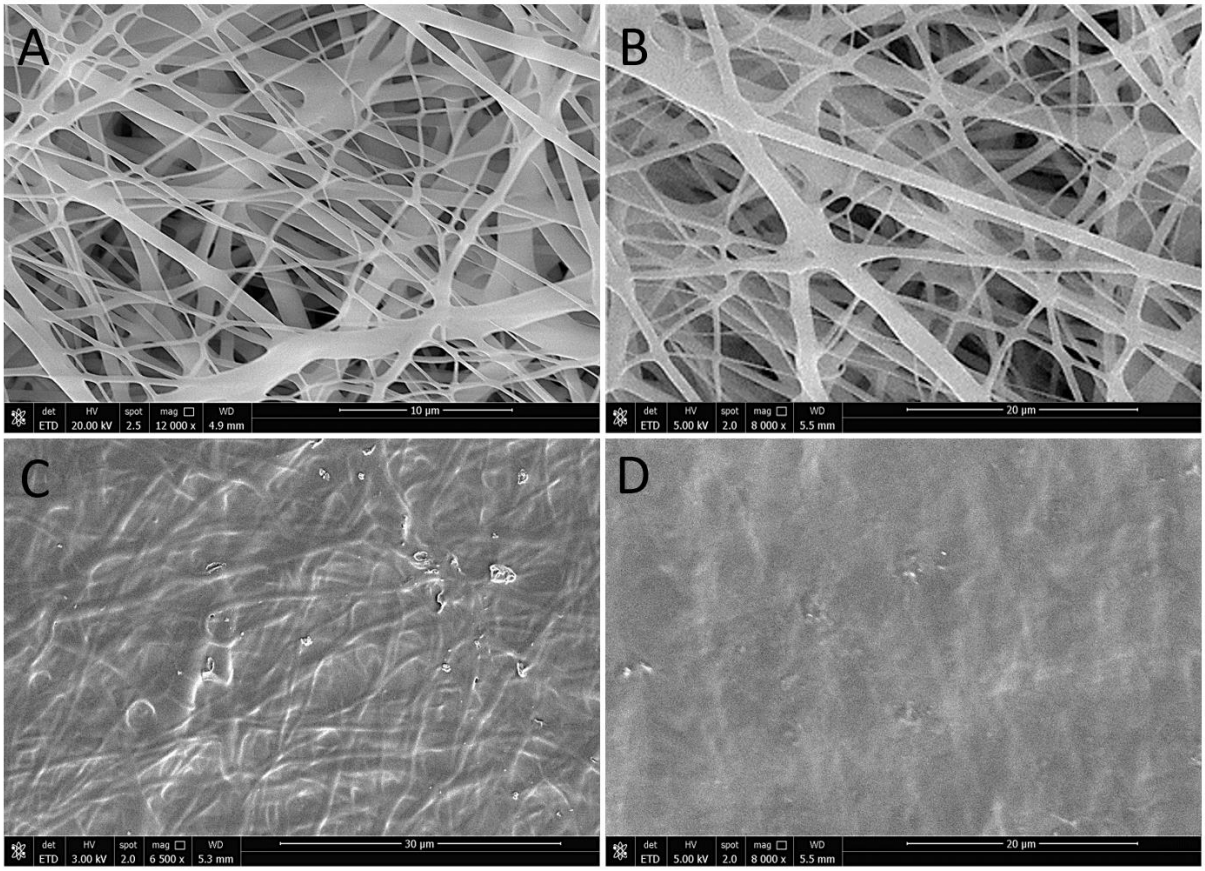

Figure 1. Scanning electron micrographs of dehydrothermally crosslinked electrospun gelatin/genipin nanofibrous construct (A) Uncrosslinked, (B) DHT crosslinked (C) DHT crosslinked in the presence of 1X PBS and (D) cell culture media.

Fig. 1A, uncrosslinked gelatin/genipin nanofibrous constructs are uniformed long smooth nanofibers. After crosslinking with 5.55:1 and 2.48:1 molar ratios of EDC/Sulfo-NHS, their corresponding SEM micrographs were acquired to evaluate the effect on fiber morphology. As illustrated in Fig. 2, the electrospun gelatin/genipin construct exhibits smooth surfaces with uniform dense network of nanofibers due to the non-zero-length crosslinking afforded by genipin to bind the gelatin network structure by bridging the free amine groups of lysine in the gelatin structure via Michael addition to form stable intermediate. This in turn elicits a nucleophilic substitution of free lysine amino groups into genipin activated ester in a secondary reaction [23]. Whilst EDC/Sulfo-NHS, a zero-length crosslinker activates carboxylic acid groups and facilitate their reaction with amine groups of lysine to result in the formation of an amide bond during crosslinking [24, 25]. The role of Sulfo-NHS activation of EDC-bound gelatin is to preserve and/or increase its water-solubility via the charged sulfonate group in order to stabilize and promote the EDC-activation of carboxylic acid residues of aspartic acids and convert them into O-acylisourea groups to form semi-stable amine-reactive NHS ester, which are less susceptible to hydrolysis [5]. The unique water content of

EDC/Sulfo-NHS (90% Ethanol, pH 6.64) crosslinking solution provided a stable crosslinking process that last for several hours (7*h*), thereby increasing the electrospun gelatin nanofibrous crosslinking efficiency. On the other hand, the activation of EDC-bound gelatin with NHS decreases its water-solubility, which can then hydrolyze within minutes or hours depending on water-content and pH of the crosslinking solution. Thus, this novel practical approach using genipin/EDC/Sulfo-NHS for the fabrication of crosslinked gelatin nanofibrous construct was employed to crosslink and stabilize the construct. Fig. 2 A and B are similar to each other where the average fiber diameter were 625 ± 231 nm and 657 ± 167 nm, for 5.55:1 and 2.48:1, respectively. It is apparent that the high molar ratio of EDC/Sulfo-NHS (Fig. 2A) results in an increase in nanofiber fusion and a decrease porosity, thereby reducing the crosslinking efficiency as the molar ratio of EDC/Sulfo-NHS increases. This may be attributed to the low concentration of Sulfo-NHS available to promote the EDC-activation of aspartic amino acids and may result in uncrosslinked nanofibrous construct as a result of the hydrolysis of the O-acylisourea group or the formation of a stable N-acylurea derivate [26].

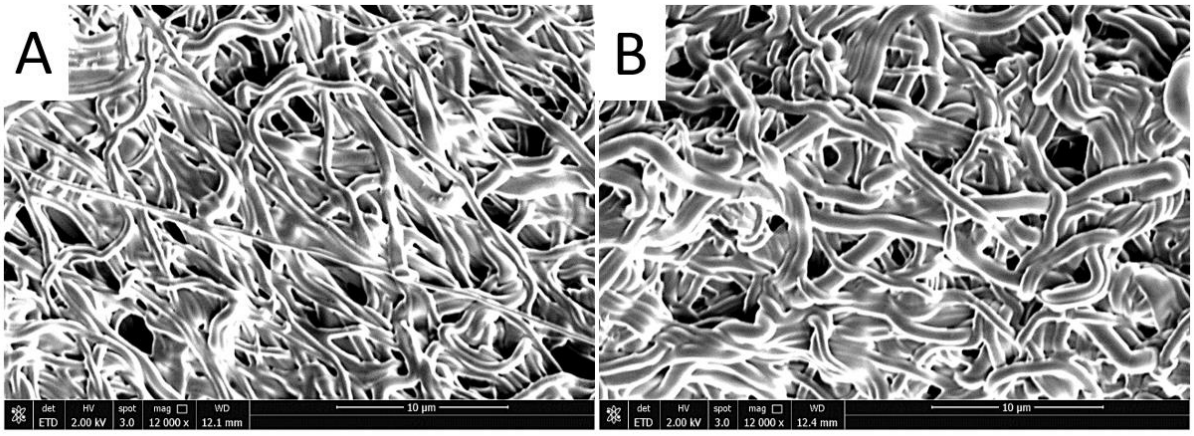

Figure 2. Scanning electron micrographs of electrospun gelatin crosslinked construct with different EDC/Sulfo-NHS crosslinker ratios (A) 5.55:1 and (B) 2.48:1.

Furthermore, samples crosslinked with 2:1 molar ratio of EDC/Sulfo-NHS have been stored at 37°*C* in 1X PBS and cell culture media for 5*h* and 5 days to assess the fiber morphology after immersion in aqueous solution. Fig. 3A-D display the SEM images of the crosslinked gelatin/genipin nanofibers soaked in 1X PBS and cell culture media for 5h and 5 days. As depicted in Fig. 3A and C, after soaking in 1X PBS, the obtained gelatin nanofibers retained similar nanofiber structure but with an increase in fiber diameter and fiber fusion. Some deposition of salt particulates was observed on the side edges of the nanofibers. The amount of salt deposition was observed to increase from 5*h* to 5 days. However, the fiber diameter on average was observed to be 723 ± 438 nm before immersion and 932 ± 217 nm after 5*h* of soaking in 1X PBS and 801 ± 295 nm after 5 days in 1X PBS. A similar observation was made with the nanofibrous construct immersed in media for 5*h* and 5 days (Fig. B and D), wherein salt and/or protein were deposited on the side edges of the nanofibrous constructs and the nanofibrous construct retained similar fiber morphology to their as-spun counterparts. However, the average fiber diameters after immersion in cell culture media were 1048 ± 344 nm and 946 ± 274 nm after 5*h* and 5 days, respectively. In both aqueous environments, the gelatin nanofibrous constructs experienced initial swelling in fiber diameter. A lower degree of swelling was observed in the samples immersed in 1X PBS due to shielding effect in 1X PBS that partially prevents the penetration of water molecules into the nanofibers. The decreased in fiber diameter after 5 days can be attributed to degradation of the nanofibers in aqueous solutions. Compared

with DHT crosslinked gelatin nanofibrous constructs, the crosslinked gelatin/genipin nanofibrous constructs demonstrated an improved retention of fiber morphology in 1X PBS and cell culture media.

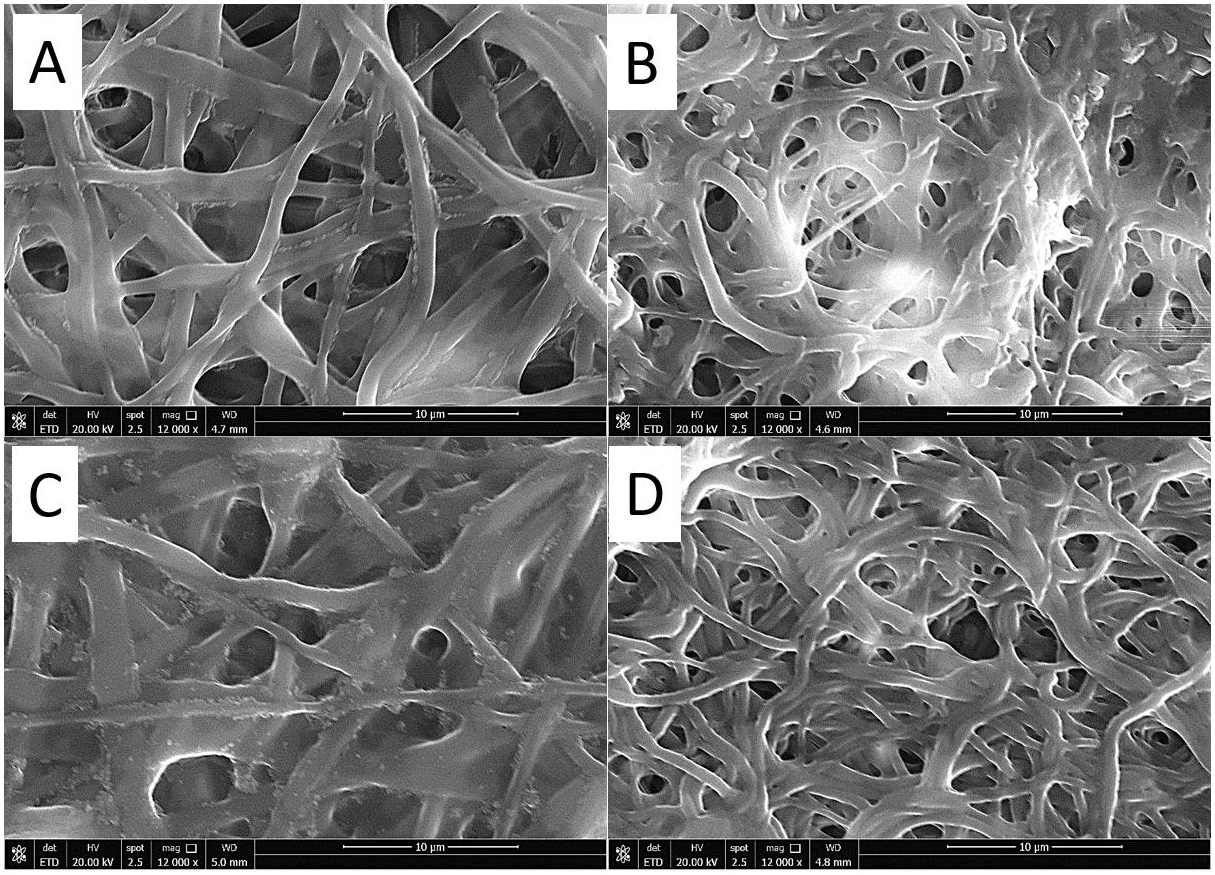

Figure 3. Scanning electron micrographs of electrospun gelatin crosslinked construct with 2:1 molar ratio of EDC/Sulfo-NHS immersed in (A) 1X PBS and (B) cell culture media for 5h (C) 1X PBS and (D) cell culture media for 5 days.

Upon disinfecting and washing the gelatin/genipin nanofibrous construct in ethanol, the morphology and structure was retained, wherein the salt residues previously observed on the side surface of the nanofibers were removed. The analysis of the fiber diameters in various concentrations of ethanol (Fig. 4) exhibited fiber diameters of 769 ± 312, 723 ± 438, 780 ± 356, and 775 ± 319 nm in the presence of 100%, 90%, 80%, and 70% ethanol, respectively. 70% ethanol is typically employed for disinfection due to its ability to effectively coagulate/denature proteins and permeates cell membranes in the presence of water molecules [27].

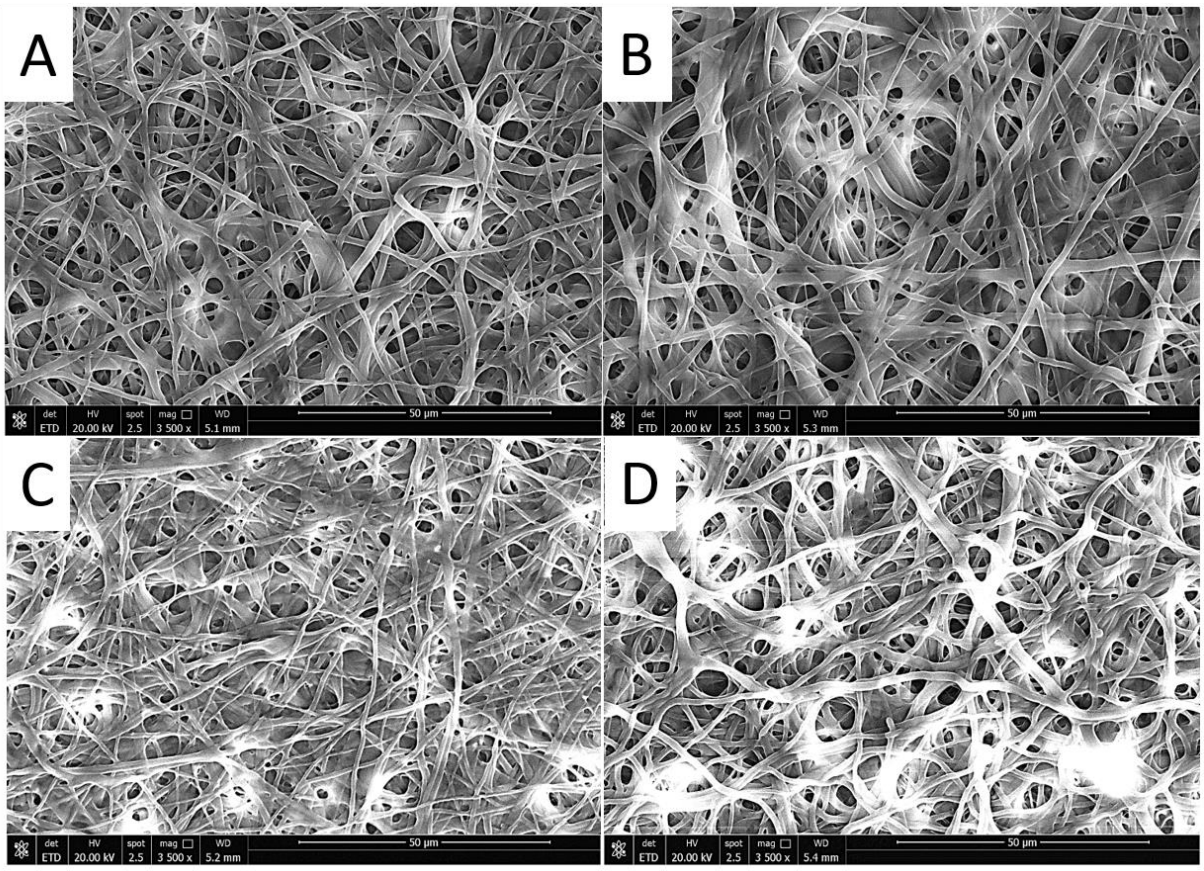

Figure 4. Scanning electron micrographs of electrospun gelatin crosslinked constructs disinfected with (A) 100, (B) 90, (C) 80, and (D) 70% ethanol.

The SEM results strongly support that the electrospinning of gelatin/genipin followed by 7*h* EDC/Sulfo-NHS crosslinking is facile, unique, and cost-effective technique that overcomes the limitations of the 24*h* glutaraldehyde vapor and the 5 day genipin crosslinking. This is a highly desirable chemical crosslinking that provides a more effective crosslinking strategy due to the use of Sulfo-NHS to stabilize the EDC-activation of the carboxylate molecules, while the genipin in the gelatin nanofibrous construct reacts with the amine groups of lysine resulting in the formation of an amide bond during crosslinking. In addition, crosslinking using this combined approach results in the retention of the morphology of gelatin nanofibrous constructs in aqueous solutions and dramatically strengthen the mechanical properties of gelatin. An additional benefit of this approach is that it enables genipin and EDC/Sulfo-NHS crosslinking to be achieved in 7*h* versus the 5 days typically required for complete genipin crosslinking. Moreover, crosslinking with genipin alone presents several drawbacks, such as opaque blue to dark blue constructs, reduced elasticity and mechanical strength.

Thermal and mechanical properties: Since a drawback of this combined approach is that the small amount of genipin integrated into the gelatin nanofibrous construct may be released upon degradation, pure electrospun gelatin nanofibrous construct was crosslinked with EDC/Sulfo-NHS (2:1 molar ratio) to achieve zero-length crosslinking. All residues generated in this crosslinking reaction are water soluble, and are removed from the construct via a rinsing/ washing step. Thermal stability of as-electrospun gelatin and gelatin/genipin nanofibrous constructs was measured using TGA in nitrogen atmosphere. Fig. 5 shows the TGA thermograms of electrospun gelatin and gelatin/genipin nanofibrous constructs before and after

crosslinking. Both of the crosslinked gelatin (curve b) and the gelatin/genipin (curve c) curves showed a similar trend of thermal stability: two weight loss peaks were observed in Fig. 5 and a rapid change in mass occurs in the range of 290 °C – 400 °C. All the samples showed an initial weight loss around 80 °C as a result of the vaporization of the moisture in the electrospun nanofibrous constructs upon heating [28].

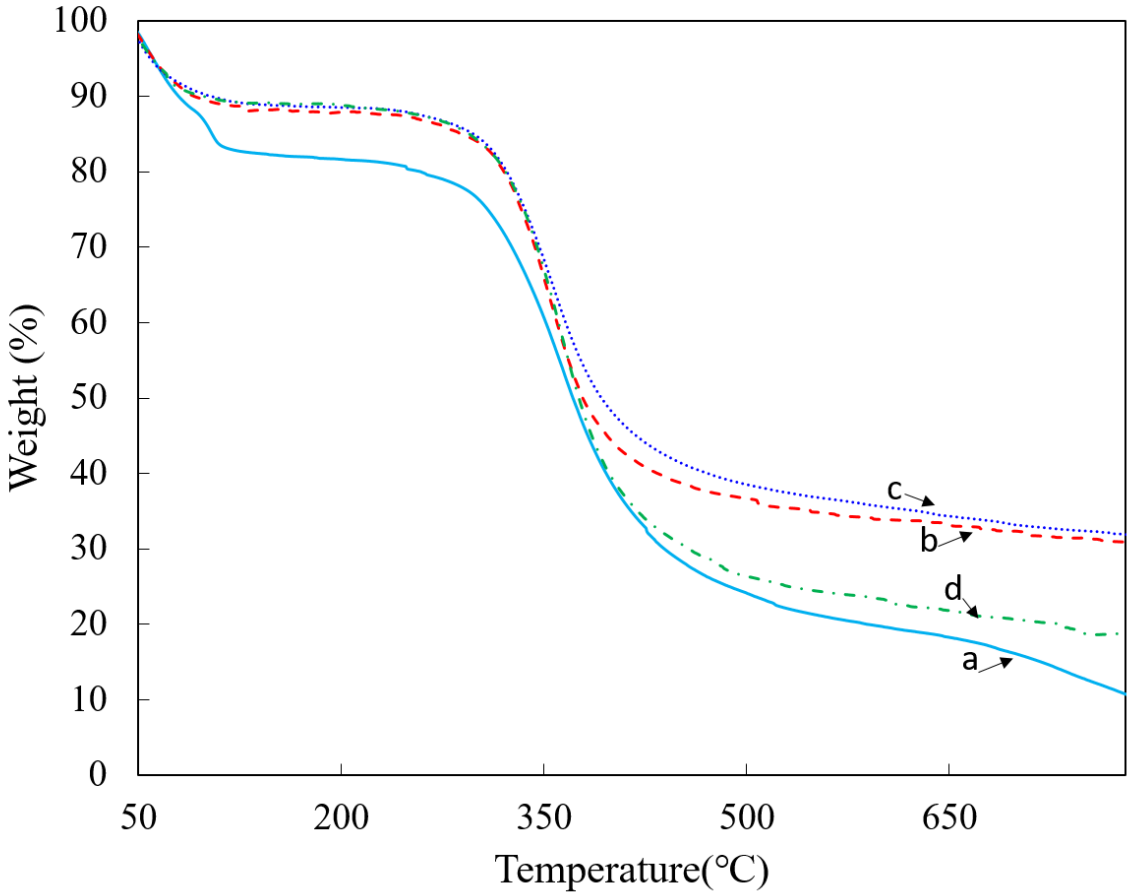

Figure 5. TGA thermograms of as-electrospun (a) uncrosslinked pure gelatin, (b) crosslinked pure chitosan, (c) crosslinked gelatin/genipin and (d) 70% ethanol treated gelatin/genipin nanofibrous constructs.

Immediately following 86 °C, there was an increased thermal stability from the uncrosslinked gelatin nanofibrous construct to the pure crosslinked gelatin nanofibrous to the crosslinked gelatin/genipin nanofibrous constructs. When the temperature is increased from 240 ℃ to 520 ℃, the weight-loss of uncrosslinked gelatin, crosslinked gelatin and gelatin/genipin are 58%, 51%, and 51% respectively, whereas the percentage residues at 800 ℃ are 9%, 31%, and 32% respectively. The increased thermal stability is attributed to the decreased mobility in the polymer chains and decrease in rate of the decomposition of the crosslinked nanofibrous constructs as a result of the homogeneous distribution of gelatin nanofibers in the constructs. The thermogram of the uncrosslinked gelatin shows decomposition temperatures at 365℃ and 520 ℃, whereas the crosslinked gelatin and gelatin/genipin thermograms exhibit decomposition temperatures at 358 ℃ and 363 ℃, respectively. Thus the decomposition profile of gelatin nanofibrous constructs provides an indication of the crosslinking process to enhance the chemical and physiochemical properties of the gelatin constructs. Upon disinfecting and washing the gelatin/genipin nanofibrous

construct in 70% ethanol, the thermal stability was reduced at 380 °C to that of the uncrosslinked gelatin and after 410 °C the stability began to increase again and hence, the differences in the respective thermal behaviors were a consequence of processing and crosslinking. Nevertheless, the thermal stability of crosslinked gelatin nanofibrous construct was increased by using genipin and EDC/Sulfo-NHS and is attributed to the presence of interchain crosslinks within the gelatin molecules and the formation of the peptide bonds involving the amino acids [29]. The TGA results indicate the crosslinking treatment has substantially enhanced the thermal stability of the as-electrospun gelatin fibers.

Mechanical characterizations were performed on both uncrosslinked and crosslinked pure electrospun gelatin nanofibrous constructs. The measurement was collected in triplicates for each material and the results were averaged together and displayed in Fig. 6. From each curve of construct type *i*, the maximum loading force ($F_{i,\max}$) was recorded and the ultimate tensile strength ($\sigma_{i,\max}$) was calculated by Eq. (2):

$$\sigma_{i,\max} = \frac{F_{i,\max}}{A_i} \qquad (2)$$

where A_i is the average cross-sectional area and the Young's modulus (E_i), ratio of stress and strain in the elastic deformation range, was calculated from the slope (α_i) of the linear portion of the load vs. displacement curve by Eq. (3):

$$E_i = \frac{\alpha_i L}{A_i} \qquad (3)$$

As depicted in Fig. 6, The crosslinked construct exhibited a different stress-strain profile compared to the uncrosslinked construct. The crosslinked construct shows an elastic modulus of 1.813 ± 0.385 GPa, which is more than 16 times greater than the uncrosslinked construct (0.108 ± 0.027 GPa). The tensile strength of the crosslinked construct reaches 16.06 ± 1.76 MPa, which is more than 8 times greater than the uncrosslinked gelatin (2 ± 0.32 MPa). A good recovery followed by a hookean linear deformation was observed with an increase in the initial modulus and tensile strength for the crosslinked construct. The mechanical behavior of crosslinked constructs and their apparent modulus are comparable with those of previous reports in the literature [25]. The crosslinked gelatin nanofibrous construct led to a substantial improvement of the mechanical properties in the tensile strength. A comparison of the mechanical properties of the measured samples with those of other electrospun materials indicate that the crosslinked gelatin construct has a lower stiffness compared to other crosslinked gelatin materials [25, 26]. In addition, an improvement in the tenacity and elongation of the crosslinked gelatin construct was observed. This improvement is attributed to the characteristics of EDC/Sulfo-NHS crosslinker to enhance crosslinking

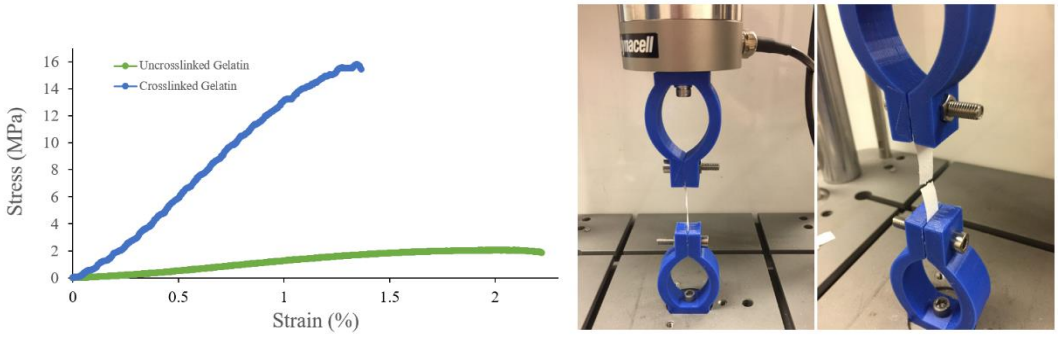

Figure 6. Tensile properties of EDC/Sulfo-NHS crosslinked pure gelatin (crosslinker ratio (2:1).

efficiency to the enhanced cross-linkage of gelatin and when used to crosslink gelatin, it acts as a reinforcement.

FTIR analysis: FTIR spectroscopy was used to study the structural characteristics of the as-electrospun gelatin and gelatin/genipin nanofibrous constructs before and after crosslinking with EDC/sulfo-NHS and treatment with 70% ethanol. All the four spectra exhibit broad band at 3100–3500 cm⁻1 due to O-H and N-H stretching (Fig. 7 insert). As shown in Fig. 7, after crosslinking, the characteristic absorption bands of the gelatin at 1638 cm^{-1} (amide I, C=O stretching), 1538 cm^{-1} (amide II, N-H bending) and 1242 cm^{-1} (amide III, C-N and N-H vibrations) shifted to lower wave numbers of 1629 cm^{-1}, 1531 cm^{-1} and 1233 cm^{-1} respectively, which indicated the crosslinking process [30n]. In the crosslinked gelatin (curve a) and gelatin/genipin (curve c) nanofibrous constructs, the absorption peak at 1093 cm^{-1} shifted to a broadened peak at 1070 cm^{-1}, which were absent in the spectrum of the uncrosslinked gelatin (curve a). These peaks are attributed to the anhydride (CO-O-CO stretching) due to the formation of carboxylic anhydride intermediate product during the EDC/NHS crosslinking process [29]. The ethanol treatment of the crosslinked gelatin/genipin nanofibrous construct did not change the chemical structure of the gelatin. Moreover, all spectra of crosslinked nanofibrous constructs under the various storage conditions were similar and exhibited the most characteristic peaks of the uncrosslinked gelatin except the appearance of broad absorption peak at 1070 cm^{-1} attributed to the stretching modes of the anhydride group upon crosslinking via EDC/Sulfo-NHS.

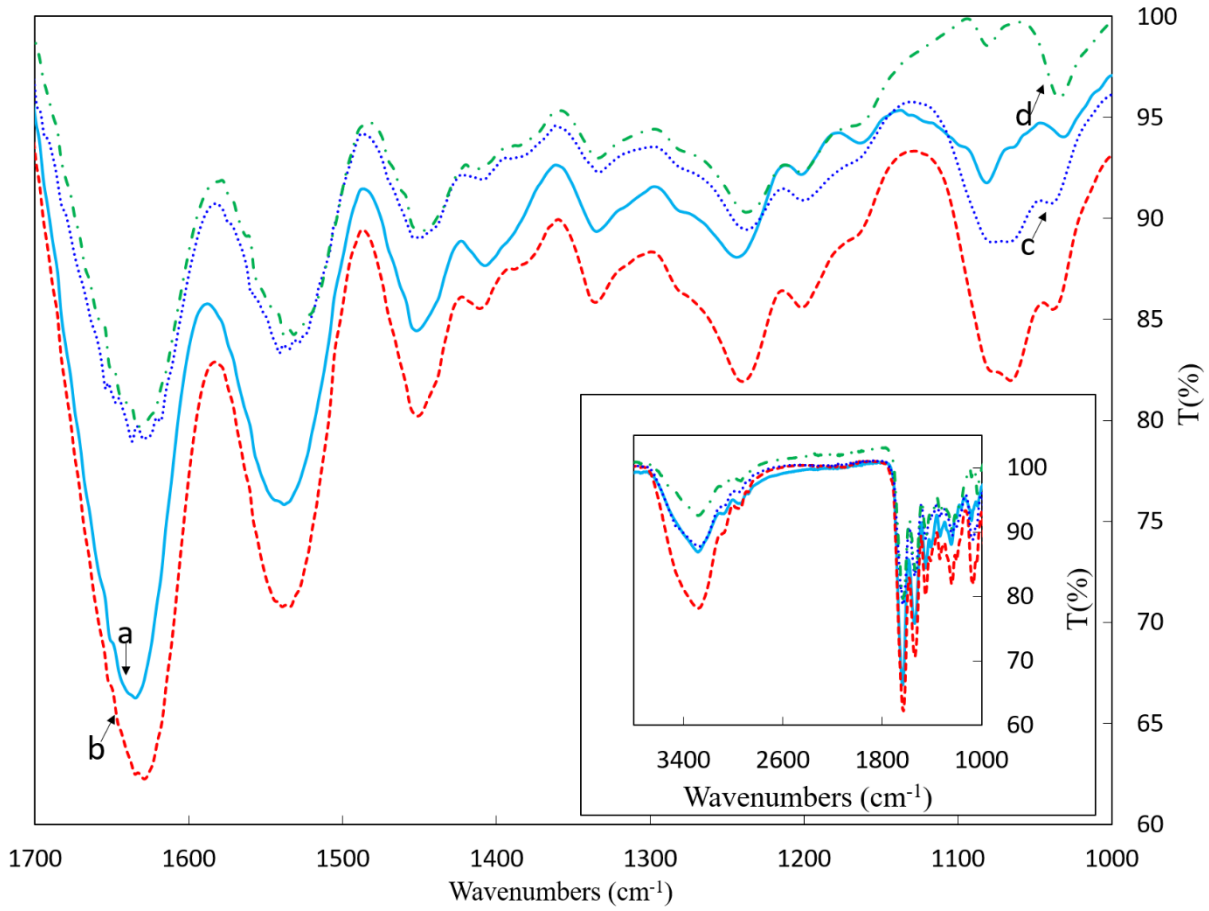

Figure 7. FTIR spectra of as-electrospun (a) uncrosslinked pure gelatin, (b) crosslinked pure chitosan, (c) crosslinked gelatin/genipin and (d) 70% ethanol treated gelatin/genipin nanofibrous constructs.

The water-insolubility of the crosslinked gelatin and crosslinked gelatin/genipin nanofibrous constructs were greatly enhanced, to such an extent that the structure of the constructs remained intact after being soaked in 1X PBS for 19 days. Fig. 8 depicts the changes in the physical appearance of the crosslinked gelatin constructs stored in 1X PBS for up to 19 days, wherein after 19 days, there was visible degradation

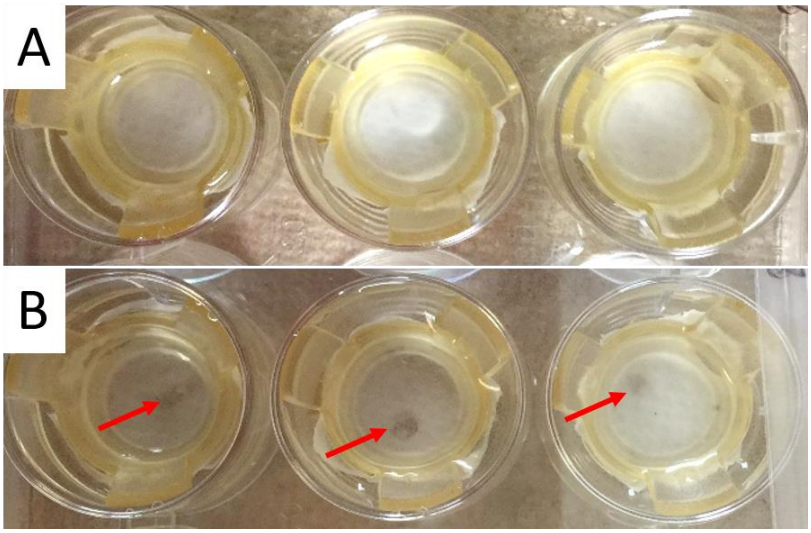

Figure 8. Optical image of the degradation of crosslinked gelatin/genipin nanofibrous constructs in 1X PBS on (A) day 1 and (B) day 19.

in the nanofiber constructs compared to their as-spun counterparts on day 1.

The degree of degradation was determined by quantifying the weight of samples crosslinked with 2:1 molar ratio of EDC/Sulfo-NHS before and after immersion in 1X PBS for 5 days to evaluate the degradation as a result of crosslinking. The samples were dried in a conventional oven at $37°C$ overnight. The evolution of weight loss was calculated by Eq. (1):

$$Weight\ Loss = \left(\frac{W_i - W_f}{W_i} \right) \times 100$$

where W_i is the initial weight of sample and W_f is the final weight after the drying period.

There was no observable degradation upon crosslinking pure gelatin nanofibrous constructs with EDC/Sulfo-NHS, wherein the fiber morphology was retained. However, there was a slight increase in the degree of degradation upon immersing the crosslinked gelatin/genipin construct in 1X PBS for 5 days. An overall weight loss of 6.13 ± 1.33% (n = 5) was observed. This result indicates that chemical crosslinking of electrospun gelatin imparts significant control over the its solubility, thereby allowing for fabrication of constructs with low degradation rates. This led to the assessment of evaluating the stability of the crosslinked pure gelatin nanofibrous constructs in dehydrate and hydrated conditions.

In order to investigate the storage stability of the crosslinked gelatin nanofibrous construct, samples were stored at 4, 22, and 37 °C (Fig. 9) in the hydrated state and -80, -20, 4, 22 °C (Fig. 10) in the dehydrated state. The morphological and thickness differences in the crosslinked gelatin nanofibrous constructs were chosen for such characterization, as they can be used to assess the different phases of degradation. The initial fiber diameter and mat thickness of the uncrosslinked pure gelatin construct were 257 ± 57 nm and 54 ± 8 μm (DAY 1), respectively. Upon EDC/Sulfo-NHS crosslinking, the fiber diameter increased to 326 ± 112 nm, whereas the construct thickness decrease to 30 ± 3 nm as the gelatin nanofibrous construct

compresses to form a compact construct and confirms the good stiffness for the crosslinked gelatin nanofibrous construct, which is in agreement with the stiffness previously discussed. Upon storage in 1X PBS, the crosslinked gelatin nanofibrous constructs stored at 4 °C were observed to retain their pristine fiber morphology and diameter (605 ± 125 nm) on day 14 to its counterpart on day 1 (596 ± 120 nm). However, the fiber diameter decreased slightly to 567 ± 128 nm on day 30. This is attributed to the low relative humidity (17%) and the refrigeration temperature to slow down chemical reactions that accelerate the degradation process, thereby preserving the crosslinked pure gelatin fibers for long periods. The samples stored in PBS at room temperature (22 °C) with a relative humidity of 20% experienced initial swelling in fiber diameter and an increase in fiber fusion, wherein the fibers began to degrade in a biphasic fashion between day 14 and 30. This is attributed to initial dissolution of small diameter fibers followed by degradation of the larger diameter fibers at a warmer temperature [31]. In a humidified CO_2 incubator (37 °C and 5% CO_2), the hydrated crosslinked gelatin nanofibrous construct was not able to retain its fiber morphology after day 14 as a result of the rapid degradation process at an elevated temperature. However, it is important to note that complete dissolution of the construct was not observed.

In the dehydrated state (Fig. 9), the crosslinked gelatin nanofibrous constructs stored at -20 and -80 °C were observed to retain their fiber morphology and diameter (on average 358 ± 97 nm) on day 14 to its counterpart on day 1 (326 ± 112 nm). However, the fiber diameter decreased slightly to 340 ± 94 nm on day 30. The thickness of the construct was retained at 30 ± 3 μm from day 1 through 30. It is apparent that there was no noticeable change in the fiber morphology and construct thickness at -20 and -80°C. At a storage temperature of 4 °C, the crosslinked gelatin nanofibrous construct exhibited similar morphology and fiber diameters to their as-crosslinked counterparts on day 1, wherein the fiber diameter increased from 418 ± 119 to 436 ± 120 nm and the thickness of the construct decreased slightly from 29 ± 2 to 28 ± 2 μm, from 14 to 30 days, respectively. The samples stored at room temperature (22 °C) with a relative humidity of 20% experienced initial swelling and a slight increase in fiber fusion. The nanofiber diameter decrease from 466 ± 106 to 433 ± 116 nm and the thickness of the construct remained relatively the same 21 ± 7 μm from 14 to 30 days, respectively. Overall, the structure and morphology of the samples were retained at all storage conditions; however, some fibers were observed to fuse together at 22 °C, which is attributed to the slightly higher relative humidity. The optimum storage condition was observed at 4 °C, -20°C and -80°C where the crosslinked nanofibrous gelatin constructs were highly stable with improved fiber retention after 30 days.

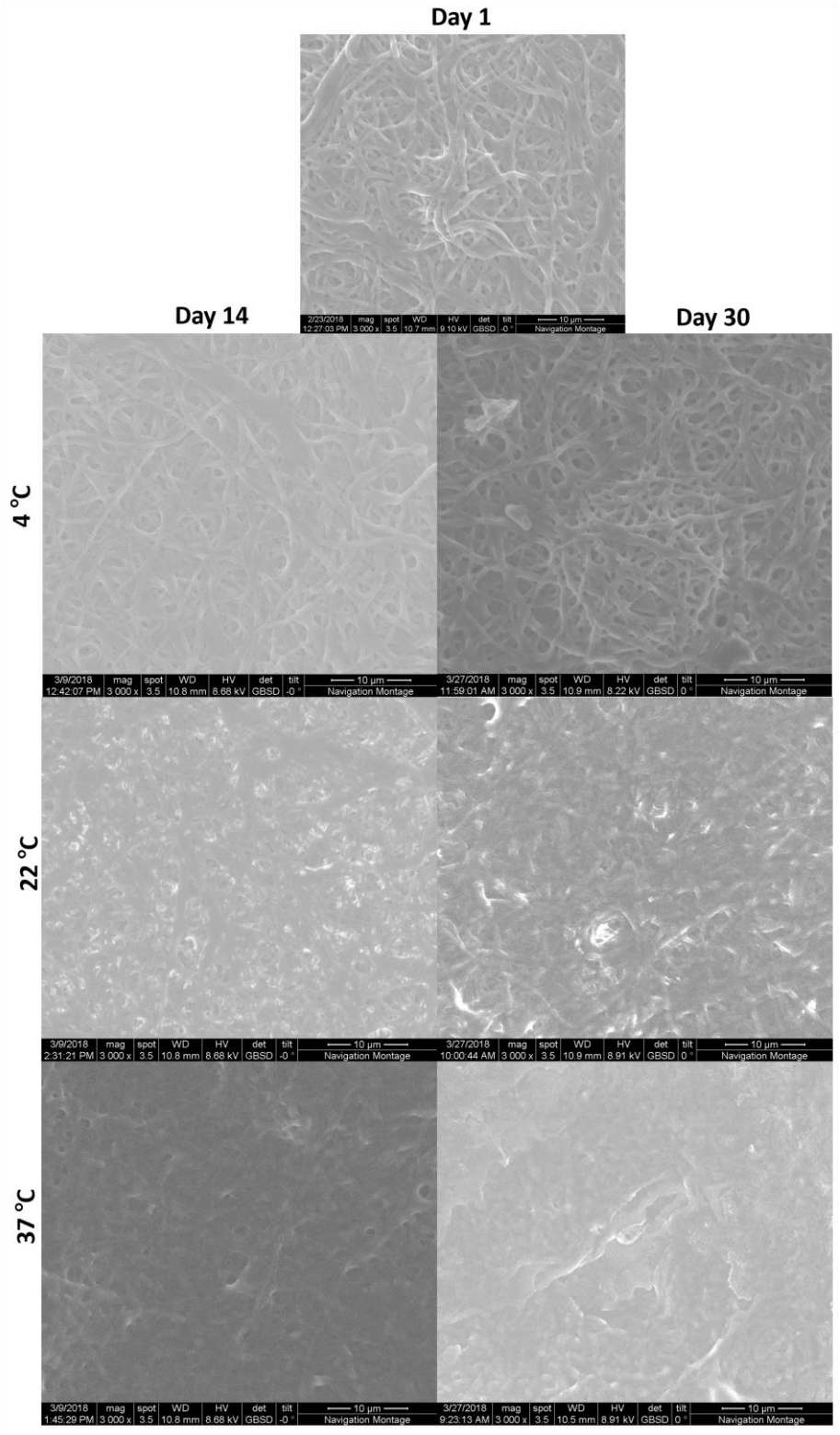

Figure 9. Scanning electron micrographs (E-SEM) of crosslinked pure gelatin nanofibrous constructs stored at various temperatures in 1X PBS for up to 30 days.

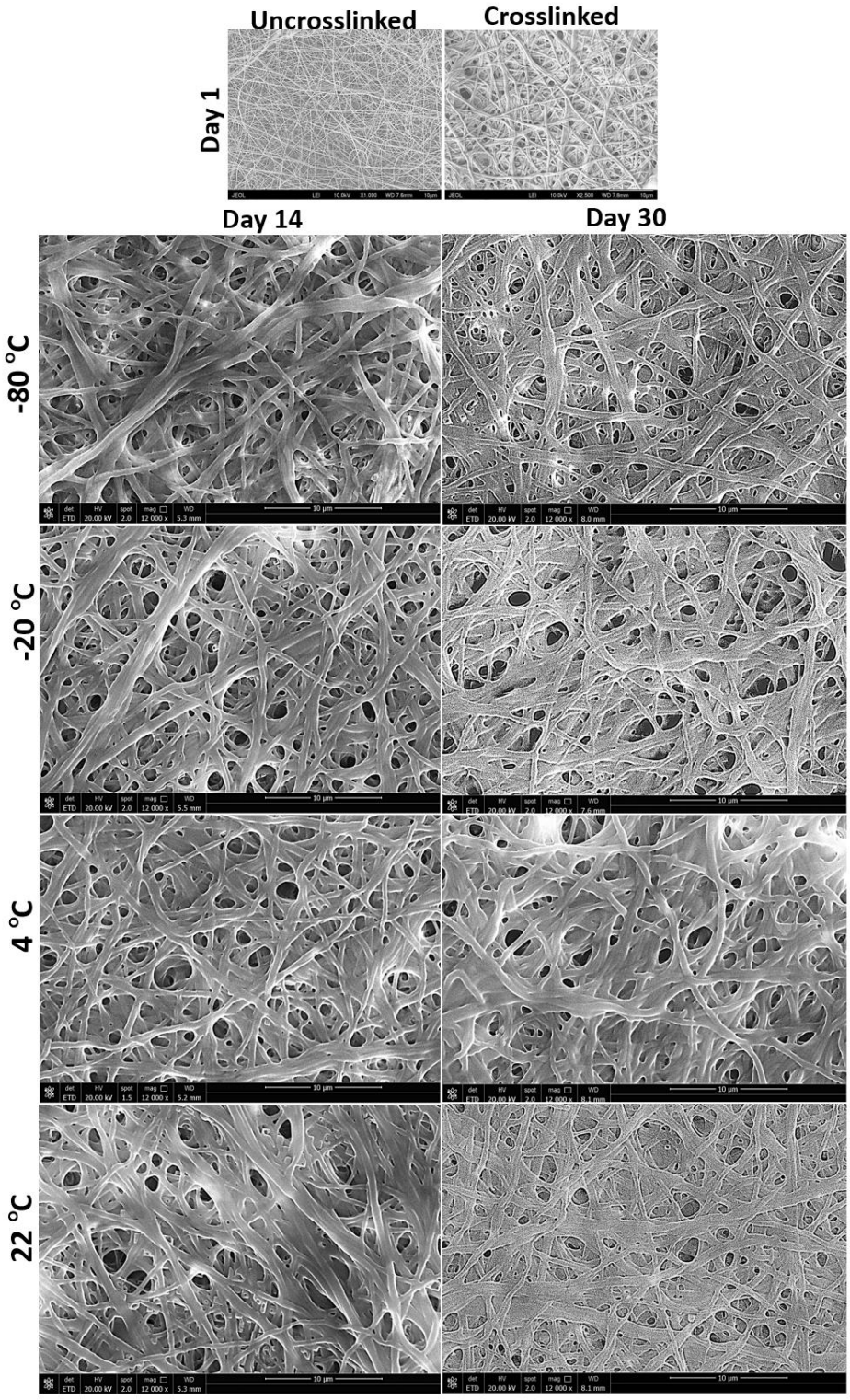

Figure 10. Scanning electron micrographs of dehydrated crosslinked pure gelatin nanofibrous constructs store at various temperatures for up to 30 days.

Biocompatibility Assays: The biocompatibility of the EDC/Sulfo-NHS crosslinked electrospun pure gelatin construct was evaluated using PC-12 cells as an in vitro model. PC-12 cells were double-stained for fluorescence using calcien-AM (green) and EthD-1 (red), respectively. Figure 11A-B shows the live/dead assays of cell viability after 48*h.* The results show that the cells grow normally on the crosslinked gelatin construct as well as in the control well for a period of 2 days. The prepared nanofibers exhibited very good cell viability of 90% with the PC-12 cells grown in clusters on top of the nanofibrous construct compared to the control sample viability of 95%. It is important to note that the 3D nanometer sized fiber organization promotes cell proliferation by mimicking the topography and high surface area exhibited by extracellular matrix (ECM). This verifies that the use of EDC/Sulfo-NHS as a zero-length crosslinker was very little or no cytotoxic effect on the cells. Indeed, the EDC/Sulfo-NHS residues were removed during the washing stage. Morphological analysis indicated that the PC-12 cells attached and spread on the crosslinked electrospun gelatin nanofibrous construct. Fig. 9D shows the PC-12 cell attachment and growth on crosslinked electrospun gelatin nanofibrous construct after cell fixation with DAPI (blue) to highlight the nuclei. It can be seen that PC-12 cells adhered and spread on the surface of the crosslinked gelatin nanofiber and these cells interacted and integrated well with the surrounding nanofibers. The crosslinked pure gelatin nanofibrous construct has potential application in the pharmaceutical and medical fields for creating 3D scaffolds for drug delivery and tissue engineering [32-34].

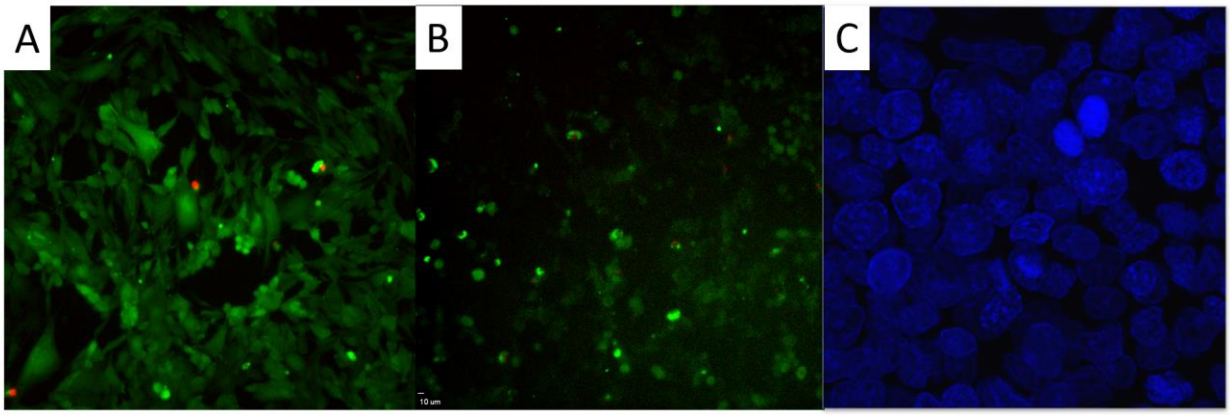

Figure 11. Representative confocal images of Live-Dead assay on PC-12 cells was performed after 2 days. Fluorescence staining of viable cells (green) and dead cells (red) on (A) control surface, (B) crosslinked pure gelatin and (C) DAPI staining (blue) to highlight the nuclei.

Conclusions

Gelatin/genipin nanofibers and pure gelatin nanofibers with high aspect ratio structure were successfully fabricated using a single solvent electrospinning technique. The morphological results showed smooth surface nanofibers without any beads and exhibit smooth and uniform diameters along their lengths. These gelatin nanofibrous constructs were successfully crosslinked with EDC/Sulfo-NHS to increase aqueous stability and to achieve non-zero length and zero length crosslinking. This resulted in increased mechanical strength and elasticity in the crosslinked gelatin nanofibrous construct. The increase mechanical properties in the crosslinked nanofibrous construct can lead to the broad range of storage condition, wherein crosslinked nanofibrous exhibit incredible storage stability. The storage stability of dehydrated crosslinked pure gelatin nanofibers showed optimum storage environment at 4 °C to -80 °C. In hydrated conditions, the sample at 37 °C showed faster degradation. In addition, the crosslinked gelatin nanofibrous construct showed PC-12 cells exhibited good attachment and proliferation on the gelatin nanofiber scaffolds. Live/Dead assay confirmed nanofibers have cell viability property and cytocompatibility. The crosslinked

gelatin offers unique capabilities in providing a microenvironment for cell growth and allowing continuous formation of 3D cell networks without toxicity during in vitro use. The collective results suggest that EDC/Sulfo-NHS crosslinking could eventually find a wide range of applications in 3D natural protein scaffolds in cell based assays and tissue engineering in vitro.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

electrospinning, gelatin; storage stability; EDC/Sulfo-NHS crosslinking; genipin

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