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Contributed Paper

Electrospun Zein Fibrous Membranes Using Glyoxal as Cross-Linking Agent: Preparation, Characterization and Potential for Use in Biomedical Applications

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ABSTRACT

In the present contribution, ultra-fine zein fibers were successfully prepared by electrospinning. The diameters of these fibers ranged between 0.32 and 1.10 μm. Cross-linked electrospun zein fiber mats were prepared by adding varying quantities of glyoxal aqueous solution (i.e., 0, 5, 10 and 15% v/v based on the volume of the zein solution) into the base zein solution (i.e, 40% w/v). Mechanical properties of the electrospun zein fiber mats were evidently improved by the cross-linking with glyoxal. The fibrous nature of the cross-linked materials was preserved even after submersion in distilled water at room temperature (i.e., 25±1°C) or 37°C for 5 d. The potential for use of the cross-linked electrospun zein fiber mats as scaffolding materials for cell/tissue culture was assessed with indirect cytotoxicity evaluation with mouse fibroblasts (L929) and direct culture with human foreskin fibroblasts (HFF). It was found that an increase in the amount of glyoxal used to cross-link the materials caused the cross-linked materials to be more toxic, but this can be alleviated by pre-treatment of the cross-linked electrospun zein fiber mats in a glycine aqueous solution. Finally, the culture of HFF on some of the cross-linked fiber mat samples for 5 d showed that the cells grew well on the materials.

Keywords: electrospinning, zein, fibrous scaffolds.

1. INTRODUCTION

Electrospinning is an established method for producing fibrous membranes with diameters of the underlying fibers ranging from less than ten micrometers down to tens of nanometers [1]. The principle of the process involves the application of a strong electric

field across a finite distance between a conductive capillary, connected to a reservoir of a polymer solution, and a collecting device. Under a high enough electric field, the partially-spherical shape of a pendant droplet of the polymer solution at the capillary tip is deformed into a conical shape. If the electric field exceeds a threshold value, electrical forces overcome that of the surface tension, resulting in an ejection of the polymer solution. The ejected, charged stream of the polymer solution (i.e., charged jet) moves towards the collecting device, resulting in the deposition of ultra-thin polymeric fibers on the collecting device as a non-woven membrane [1]. Parameters affecting the morphology of the fibers are, for examples, solution properties (e.g., viscosity, conductivity, surface tension, etc.), solution feed rate and electric field [2,3]. Due to the simplicity of the technique and the unique characteristics of the obtained fibers, these fibrous materials have been heavily explored for various biomedical applications, including wound dressings [4,5], carriers for drug delivery [6-8], and scaffolding materials for cell/tissue culture [9-13].

Zein is a major storage protein, making up to about 45-50% of the total proteins found in maize. The molecular structure of zein is helical wheel conformation in which nine homologous repeating units are arranged in an anti-parallel manner which is stabilized by hydrogen bonds [14]. Zein exhibits various interesting properties, such as toughness, flexibility, compressibility, glossiness, hydrophobicity, resistance to microbial attack [15] and antioxidant activity [16-18]. The proposed uses of zein in biomedical applications are as carriers for drug delivery [19-21], food packaging [22] and scaffolding materials for cell/tissue culture [23,24]. Miyoshi et al. [25] were the first group to report successful electrospinning of zein from its solutions in 80% w/w ethanol aqueous solution. Yao et al. [26] reported successful electrospinning of zein from its solutions in 70-90% v/v ethanol aqueous solutions. A more thorough study on the electrospinning of zein from its solutions in various ethanol-based solutions was reported by Torres-Giner et al. [27], who investigated a number of parameters (i.e., polymer concentration, solvent content, solution flow-rate, applied potential, needle tip-to-collector distance and pH) that affected the electrospinning of the polymer. Cross-linking of electrospun zein fiber mats from zein solutions in various ethanol aqueous solutions with hexamethylene diisocyanate (HDI) was reported by Yao et al. [26] Selling et al. [28] used glutaraldehyde as the cross-linking reagent to improve the physical properties and solvent resistance of electrospun zein fiber mats. The use of citric acid as the cross-linking reagent for electrospun zein fiber mats and sodium hypophosphite monohydrate as the catalyst was reported by Xu et al. [29]

In the present contribution, ultra-fine zein fiber mats were prepared by electrospinning. The effects of solution concentration, applied electrical potential and collection distance on morphological appearance of the obtained fibers and on the size of the individual fiber segments were investigated by scanning electron microscopy (SEM). To improve stability and mechanical integrity of the fiber mats in an aqueous medium, the electrospun zein fiber mats were cross-linked with glyoxal. The potential for use of the neat and the cross-linked zein fiber mats as fibrous membranes for various biomedical applications, such as wound dressings, was evaluated with murine fibroblasts (L929) and human foreskin fibroblasts (HFF).

2. EXPERIMENTAL DETAILS

2.1 Materials

Zein powder (M_r = 25,000-29,000 g·mol^{-1})

was purchased from Fluka Chemika/Biochemika (Switzerland). Ethanol (80% v/v aqueous solution; Carlo Erba, Italy) was used as the solvent. Glyoxal (40% v/v aqueous solution; Fluka Chemika/Biochemika, Switzerland) was used as the cross-linking agent. All other chemicals were of analytical reagent grade and used as received.

2.2 Preparation of Neat and Cross-Linked Zein Fiber Mats

To prepare the neat electrospun zein fiber mats, zein solutions in the aqueous ethanol solution were prepared at the concentrations of 30 to 45% w/v. Prior to electrospinning, the as-prepared spinning solutions were characterized for their viscosity and conductivity using a Brookfield DV-III programmable viscometer and a SUNTEX conductivity meter, respectively. Each of the as-prepared spinning solutions was later contained in a 5-mL glass syringe, the open end of which was connected to a blunt 20-gauge stainless steel hypodermic needle (OD = 0.91 mm), used as the nozzle. A Gamma High Voltage Research D-ES30PN/M692 power supply was used to generate a DC electrical potential in the range of 15 to 25 kV. The electrical potential of positive polarity was supplied to the solution across a finite distance between the tip of the needle and a sheet of Al foil wrapped around a stationary plastic backing, used as the collecting device, ranging between 10 and 20 cm (i.e., collection distance). The solution feed rate was maintained by a syringe pump at 1.5 $mL \cdot h^{-1}$ and the collection time was fixed at about 5 min.

To prepare the cross-linked electrospun zein fiber mats, the 40% w/v zein solution in the ethanol aqueous solution was mixed with varying amounts of the glyoxal aqueous solution (i.e., 0, 5, 10 and 15% v/v based on the volume of the zein solution). Prior to electrospinning, each of the spinning solutions was characterized for their viscosity and conductivity using the viscometer and the conductivity meter, respectively. Electrospinning of the as-prepared zein solutions with or without the presence of the glyoxal aqueous solution was carried out at a fixed electrical potential of 20 kV over a fixed collection distance of 15 cm onto a sheet of Al foil wrapped around a home-made rotating cylinder (rotational speed ≈ 40 rpm), used as the collecting device. The solution feed rate was also fixed at 1.5 $mL \cdot h^{-1}$ and the collection time was fixed at about 20 h. The electrospun fiber mats were dried in an oven at 60°C for 12 h. Hereafter, ZP, ZP5, ZP10 and ZP15 are used to refer to the electrospun zein fiber mats that had been fabricated from the zein solutions containing 0, 5, 10 and 15% v/v of the glyoxal aqueous solution, respectively.

2.3 Characterization of Neat and Cross-Linked Zein Fiber Mats

Morphological appearance of both the neat and the cross-linked electrospun zein fiber mats was observed by a JEOL JSM-6400 scanning electron microscope (SEM). The fiber mat specimens were coated with a thin layer of gold prior to SEM observation. Diameters of the individual fiber segments were measured directly from the SEM images using a SemAphore 4.0 software, with the average values being calculated from at least 100 measurements. In the cases where beaded fibers were obtained, the average number of beads per unit area (i.e., the bead density) and the average diameters (i.e., only measured on the fiber segments between beads) of the beaded fibers were calculated from measurements on SEM images of 3,500x and 1,000x magnifications, respectively.

Mechanical properties in terms of the tensile strength, Young's modulus and elongation

at break of both the neat and the cross-linked electrospun zein fiber mats were investigated using a Lloyd LRX universal testing machine (gauge length = 50 mm and crosshead speed = 20 mm·min^{-1}). The fiber mats of about 70±10 mm in thickness were cut into rectangular-shaped specimens (10 mm × 100 mm). For each group of fiber mat samples, about 5 specimens were tested and the results were reported as average values.

To assess the stability of the fiber mats in maintaining their fibrous structure after submersion in an aqueous medium, both the neat and the cross-linked electrospun zein fiber mat specimens (cut into circular discs of about 1.4 cm in diameter) were submerged in distilled water at either room temperature (i.e., 25±1°C) or 37°C for either 1 or 5 d. At a given time point, the morphology of the dried specimens was observed by SEM.

2.4 Cytotoxicity Evaluation

Indirect cytoxicity evaluation of both the neat and the cross-linked electrospun zein fiber mats was conducted in adaptation from the ISO 10993-5 standard test method, using mouse fibroblasts (L929). Both the neat and the cross-linked electrospun fiber mats were cut into circular discs (about 23 mm in diameter and about 60±5 mm in thickness). These specimens were divided into two groups. The specimens in the first group were tested in their native form, while those in the second group were tested after they had been treated with 0.1 M glycine aqueous solution. Prior to further investigation, the specimens were sterilized with UV radiation for about 1 h. Extraction media were then prepared by immersing the disc specimens in a serum-free medium [SFM; containing Dulbecco's modified Eagle's medium (DMEM), 1% L-glutamine, 1% lactabumin and 1% antibiotic and antimycotic formulation (containing penicillin G sodium, streptomycin sulfate, and amphotericin B)] in wells of a 24-well tissue-culture polystyrene plate (TCPS; Biokom Systems, Poland) for either 3 or 7 d. L929 were seeded at 40,000 cells/well in serum-containing DMEM for 16 h to allow cell attachment. The cells were then starved with SFM for 24 h, after which time the medium was replaced with an extraction medium. The cells of the control group were incubated with fresh SFM. After 24 h, the viability of the cells was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (see Supplementary information).

2.5 Morphological Observation of Cultured cells

Human foreskin fibroblasts (HFF; at the density of 20,000 cells/well) were cultured on the cross-linked electrospun zein fiber mats (i.e., ZP10) at 37°C. After 5 d of cell culturing, morphological appearance of the cells was observed by SEM. After removal of the culture medium, the cell-cultured specimens were rinsed with PBS twice and the cells were then fixed with 3% gluteraldehyde solution [diluted from 50% glutaraldehyde solution (Electron Microscopy Science, USA) with PBS] at 500 μL/well. After 30 min, they were rinsed again with and kept in PBS at 4°C. After the cell fixation, the specimens were dehydrated in graded ethanol solutions (i.e., 30, 50, 70 and 90%, respectively) and in pure ethanol for about 2 min at each concentration. The specimens were then dried in 100% hexamethyldisilazane (HMDS; Sigma, USA) for 5 min and later in air after the removal of HMDS. After completely dried, the specimens were mounted on SEM stubs, coated with gold, and observed by SEM.

2.6 Statistical Analysis

Data were presented as means ± standard

errors of means (n = 3). A one-way ANOVA was used to compare the means of different data sets, and statistical significance was accepted at a 0.05 confidence level.

3. RESULTS AND DISCUSSION

As mentioned, Miyoshi et al. [25] were the first group to report successful electrospinning of zein from its solutions in 80% w/w ethanol aqueous solution. The effects of the concentration of the zein solutions (i.e., 18-25 wt.%) and the applied potential (i.e., 15 and 30 kV over a fixed collection distance of 10 cm) on morphology and size of the electrospun zein fibers were reported. At 15 kV, electrospinning of the zein solutions with concentrations greater than about 21 wt.% produced smooth and ribbon-like fibers. At 30 kV, predominantly smooth and ribbon-like fibers were obtained when the concen-tration of the zein solutions was greater than about 18 wt.%. The diameters of the obtained fiber segments were reported to be about 1 μm and 700 nm, respectively [25]. Yao et al. [26] studied the electrospinning of zein from its solutions in various ethanol aqueous solutions. The effects of the concentration of the zein solutions (i.e., 10-50% w/v) and the ethanol/water ratio of the ethanol aqueous solutions (i.e., 70:30, 80:20, and 90:10 v/v) on morphology and size of the electrospun zein fibers were reported. At 70:30 v/v of the ethanol aqueous solution, smooth and ribbon-like fibers were obtained from 30-50% w/v zein solutions, with the diameters of these fibers ranging between about 1 and 6 μm. It was also observed that increasing the ethanol content in the solvent system caused the resulting fibers to be more brittle, likely a result of the self-assembling of the protein during solvent evaporation [26].

Recently, Torres-Giner et al. [27] studied various parameters that affected the morphology and size of the electrospun zein fibers. These parameters are solution concentration, ethanol content in the ethanol aqueous solution used as the solvent system, solution flow-rate, applied potential and collection distance. They found that fibers were formed over the concentration range of 25-50 wt.%. During the initial increase in the concentration of the zein solutions between 25 and 33 wt.%, the change in the fiber diameters was low, with the values being about 200 nm on average. Further increase in the solution concentration to 42 and 50 wt.% resulted in a dramatic increase in the fiber diameters, with the final values being about 1 μm on average. With regards to the effects of the ethanol content, the solution flow-rate and the applied potential, an initial increase in these parameters (i.e., 50-80% w/w for the ethanol content, 0.1-0.46 $mL \cdot h^{-1}$ for the flow-rate and 7-11 kV for the applied potential) did not have a strong influence on the diameters of the obtained fibers. Further increase in the values of these parameters resulted in an observed increase in the diameters of the obtained fibers. Similarly, an initial increase in the collection distance in the range of 5-12.5 cm did not have a strong effect on the diameters of the obtained fibers. Further increase in the values of this parameter, however, resulted in an observed decrease in the diameters of the obtained fibers [27].

Prior to electrospinning, certain properties such as shear viscosity and electrical conductivity of the zein spinning solutions were measured and the results are summarized in Table 1. Here, the concentration of the ethanol aqueous solution used as the solvent system for zein was fixed at 80% v/v. According to Table 1, an increase in the concentration of the zein solutions from 30 to 45% w/v resulted in a monotonous increase in the shear viscosity. The increase in the shear viscosity of the solution with an increase in the solution concentration was due to the increased

Table 1. Shear viscosity and electrical conductivity of the as-prepared zein solutions at 25°C (n = 3).

Solution concentration (wt.%)	Shear viscosity (mPa s)	Electrical conductivity (μS cm^{-1})
30	120 ± 1	884 ± 1
35	188 ± 1	859 ± 1
40	289 ± 2	850 ± 1
45	491 ± 1	815 ± 1

likelihood for chain entanglements [30]. An increase in the solution concentration, on the other hand, resulted in a monotonous decrease in the electrical conductivity. As pointed out by a number of researchers [1,3], the electrical forces that influence the ejection and the stretching of the ejected, charged jet are governed by the amount of unassociated charges within a jet segment. These unassociated charges, believed to be carried over by water, are in the form of residual ionic impurities [1,3], provided that none of the major substances in the spinning solution did not undergo a dissociation reaction into ionic species upon subjection to a high electrical potential. An increase in the solution concentration caused the amount of the solvent (i.e., 80% v/v ethanol aqueous solution), hence that of water, to decrease. The gradual decrease in the amount of water resulted in a hypothetic decrease in the amount of the unassociated charges, hence the observed reduction in the electrical conductivity. Notwithstanding, since the amount of water in the ethanol aqueous solution was only 20% v/v, the decrease in the electrical conductivity of the solutions was not significant.

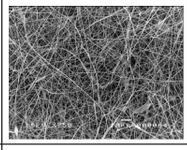
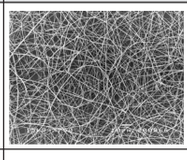
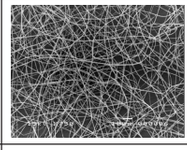
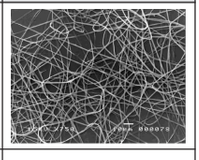
To obtain the condition that resulted in the electrospun zein fiber mats with optimized diameters, the zein solutions with concentrations in the range of 30 to 45% w/v were electrospun under applied potentials in the range of 15 to 25 kV and collection distances in the range of 10 to 20 cm for 5 min. Table 2 shows representative SEM images of the electrospun fiber mats from the zein solutions that had been fabricated under the applied potential and the collection distance of 20 kV and 15 cm, respectively. Notwithstanding, the SEM images of the electrospun fiber mats from the zein solutions that had been fabricated under all of the investigated conditions are provided as Supplementary information. According to the obtained results, the diameters of the electrospun zein fibers that had been obtained under these conditions ranged between 0.32 and 1.10 μm. For the zein solutions with the concentrations lower than 35% w/v, beaded fibers were the common features. For given values of the applied electrical potential and collection distance, increasing concentration of the zein solutions caused the diameters of the fibers to increase. For the beaded fibers, an increase in the concentration of the zein solutions resulted in decreased tendency for bead formation. For given values of the solution concentration and the collection distance, an increase in the applied electrical potential resulted in an increase in the fiber diameters. For given values of the solution concentration and the applied electrical potential, an increase in the collection distance generally resulted in a reduction in the fiber diameters, except for the fibers that had been obtained from the 45% w/v zein solution.

The results were in general accordance

with that previously reported by Torres-Giner et al. [27] Increasing the solution concentration to a value that is beyond a critical, chain-overlapping concentration results in extensive chain entanglements that are enough to prevent the total break-up of the ejected, charged jet into discrete drops [30]. Further increase in the solution concentration results in the monotonous increase in the chain entanglements that contribute directly to the observed increase in the viscosity of the solutions, hence the increase in the viscoelastic forces that counteract the electrical forces responsible for the stretching or the thinning of the charged jet segments. In addition, increasing the viscosity of the solutions causes the onset point of the bending instability to occur closer towards the collector, resulting in the reduction of the total path lengths for which the jet segments have to travel to the collector. An increase in the electrical potential also causes the onset point of the bending instability to occur closer towards the collector, which is a direct result of the increase in the electrostatic forces. On the other hand, an increase in the collection distance results in a reduction in the electrostatic forces exerting on the jet segments, causing the bending instability to occur closer to the nozzle tip, which, in turn, increases the total parth lengths for which the jet segments have to travel to the collector [30].

Since the electrospun zein fiber mats can not sustain their fibrous structure when being submerged in distilled water as well as in ethanol, cross-linking of these fiber mats is necessary. According to Table 2, the electrospinning condition that was chosen to obtain fiber mats for further investigations was 40% w/v zein solution that was electrospun under the fixed electric field of 20 kV/15 cm. This condition was selected because it was easy to spin and no discrete droplets of zein were observed during the electrospinning. The cross-linked electrospun zein fiber mats were prepared by adding varying amounts of the glyoxal aqueous solution (i.e., 0, 5, 10 and 15% v/v based on the volume of the zein solution) to the 40% w/v zein solution. Previously, Yao et al. [26] reported successful cross-linking of electrospun zein fiber mats with HDI, by immersing the electrospun fiber mats in tetrahydrofuran (THF) containing 1 wt.% HDI for 10 h. Selling et al. [28] used glutaraldehyde as the cross-linking agent and the curing of the glutaraldehyde-containing

Table 2. Representative SEM images (scale bar = 5 μm; collection time = 5 min) illustrating the effect of concentration of the zein solutions on morphology of the obtained fibers at a fixed electric field of 20 kV/15 cm that had been collected for 5 min as well as average values of diameters, bead sizes, and number of beads per unit area (i.e., bead density) of the obtained fibers.

	Solution concentration (% w/v)			
	30	**35**	**40**	**45**
				
Fiber diameters (μm)	0.46 ± 0.11	0.60 ± 0.12	0.74 ± 0.13	1.05 ± 0.27
Bead sizes (μm)	1.59 ± 0.55	-	-	-
Bead density (10^{-4} μm^{-2})	7.61 ± 2.53	-	-	-

zein fiber mats was done at different temperatures from 80 to 180°C for various time intervals in order to obtain the cross-linked fiber mats with various degrees of insolubility. Here, the approach of Selling et al. [28] was followed, but glyoxal was used instead of glutaraldehyde. Prior to electrospinning, the glyoxal-containing zein solutions were characterized for their shear viscosity and electrical conductivity and the results are shown in Table 3. Clearly, the addition of glyoxal in the zein solution caused the shear viscosity to decrease and, with increasing the glyoxal content in the solutions, the shear viscosity decreased slightly. On the other hand, the electrical conductivity decreased markedly with the initial addition of glyoxal in the solution, but the property values did not changed significantly with further increase in the glyoxal content in the solutions.

Table 3. Shear viscosity and electrical conductivity of the as-prepared zein solutions (40 wt.%) with and without the presence of 0.1 M of glyoxal aqueous solution in various amounts at 25°C (n = 3).

Type of zein solution	Shear viscosity (mPa s)	Electrical conductivity ($\mu S\ cm^{-1}$)
Neat	284 ± 2	850 ± 1
With 5% v/v glyoxal	266 ± 2	929 ± 3
With 10% v/v glyoxal	253 ± 1	929 ± 1
With 15% v/v glyoxal	252 ± 1	943 ± 1

Certain mechanical properties, e.g., Young's modulus, tensile strength, and percentage of elongation at break, of the neat and the cross-linked electrospun zein fiber mats were investigated and the results are summarized in Table 4. The results indicated the dramatic improvement in the mechanical performance of the cross-linked fiber mats over that of the neat materials. Among the cross-linked materials, the initial increase in the glyoxal content used to cross-link the zein fiber mats (up to 10% v/v) resulted in the increase in all of the values of the tested mechanical properties. Further increase in the glyoxal content to 15% v/v, the property values dropped quite dramatically. This could be a result from the dilution of the zein solution in response to the addition of a larger quantity of the glyoxal aqueous solution. Clearly, the zein fiber mats that had been cross-linked at the glyoxal content of 10% v/v exhibited the greatest property values (i.e., Young's modulus = 3,560 MPa, tensile strength = 2.2 MPa, and elongation at break = 6.7%, on average). Yao et al. [26] showed that, among the various HDI-cross-linked zein fiber mats, the ones that obtained from the 40% w/v zein solution in 70:30 v/v ethanol/water showed the greatest Young's modulus and tensile strength values of 95.2 and 4.2 MPa, respectively. Clearly, the cross-linked zein membranes as obtained in this work exhibited much greater stiffness, despite the lower tensile strength value. It should be noted, however, that measurements that relate to the mechanical properties of electrospun fibrous membranes are often hampered by the wide variations of the obtained data.

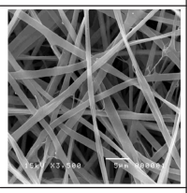
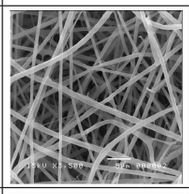
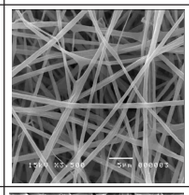
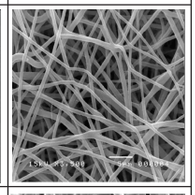
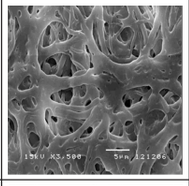
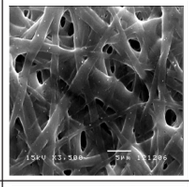
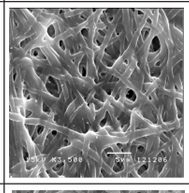
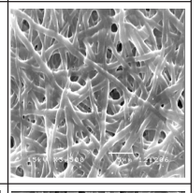
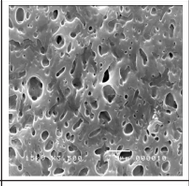
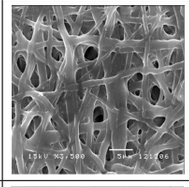
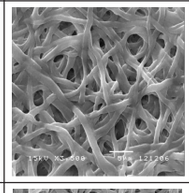
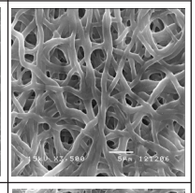
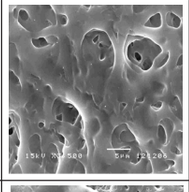
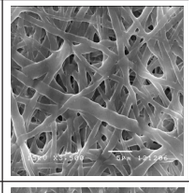
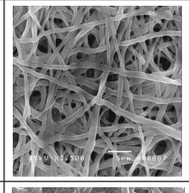
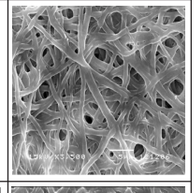
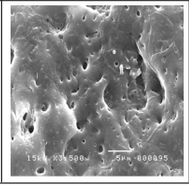
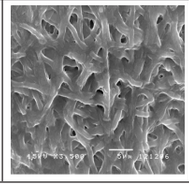
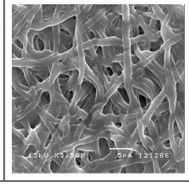
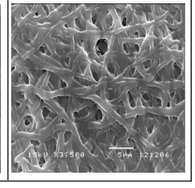
Table 4. Mechanical properties in term of Young's modulus, tensile strength and percentage of elongation at break of the neat and the cross-linked electrospun zein fiber mats (i.e., ZP, ZP5, ZP10 and ZP15 denote the fiber mats that had been obtained from the zein solutions containing 0, 5, 10 and 15% v/v of the glyoxal aqueous solution, respectively) (n = 5).

Type of the electrospun zein fiber mats	Young's modulus (MPa)	Tensile strength (MPa)	Elongation at break (%)
ZP	2,520 ± 300	0.9 ± 0.1	1.5 ± 0.4
ZP5	3,080 ± 1200	1.5 ± 0.1	5.5 ± 1.6
ZP10	3,560 ± 1600	2.2 ± 0.2	6.7 ± 2.0
ZP15	2,980 ± 500	1.3 ± 0.3	1.4 ± 0.8

The stability of both the neat and the cross-linked electrospun zein fiber mats upon their submersion in an aqueous medium needs to be determined if they are to use in certain applications that requires a contact with such medium. Some of these applications are, for examples, scaffolds for cell/tissue culture. Table 5 shows morphology of the neat and the cross-linked electrospun zein fiber mats after their submersion in distilled water at room temperature (i.e., 25 ± 1°C) and 37°C for 1 and 5 d. Apparently, the neat zein fiber mats practically lost their fibrous nature when they were submerged in distilled water at all temperatures and time points investigated, while all of the cross-linked zein fiber mats appeared to lose their fibrous nature when they were submerged in distilled water at 37°C for 5 d. Despite the improved stability in distilled water, the physical character of the cross-linked fibers changed from ribbon-like to become flattened and even fused to adjacent fibers at touching points. Evidently, the flattening and the fusing of the fibers became more obtained for the case of the neat zein fibers, as the physical character of the membranes resembled that of the solvent-cast films with embedded porous structure. To further illustrate the effect of the submersion time interval (i.e., 1, 2, 3, 4, or 5 d) on morphology of the neat and the cross-linked electrospun zein fibrous membranes, additional representative SEM images are supplied as Supplementary information.

To assess whether the neat and the cross-linked electrospun zein fiber mats could be used as scaffolding materials for cell/tissue culture, indirect cytotoxicity evaluation was carried out on these materials. Figure 1a shows the viability of mouse fibroblasts (L929) that were cultured with the extraction media that had been prepared from the neat and the cross-linked electrospun zein fiber mats for 3 d against that of the cells that were cultured with the fresh SFM. Two groups of the samples were investigated: without and with the treatment with glycine. Without the glycine treatment, the neat electrospun zein fiber mats released no substance in the level that was toxic to the cells, as the viability of the cultured cells was similar to that of the control. However, as the glyoxal content used to cross-link the materials increased, the viability of the cells decreased from about 95% (for the fiber mats that had been cross-linked with 5% v/v glyoxal) to about 42% (for the fiber mats that had been cross-linked with 15% v/v glyoxal). Upon treating the samples with glycine, the viabilities of the cultured cells were significantly greater and, even for the fiber mats that had been cross-linked with 15% v/v glyoxal, the viability of the cells appeared to be the same

Table 5. The morphology of the neat and the cross-linked electrospun zein fiber mats after submersion in distilled water at room temperature (i.e., 25 ± 1°C) and 37°C for 1 and 5 d.

Temperature (°C)	Time (day)	Materials			
		ZP	ZP5	ZP10	ZP15
	0				
25	1				
	5				
37	1				
	5				

as that of the control. Based on the obtained results, the effect of glycine may be two-fold. Firstly, the amino group of glycine helps to block the unreacted aldehyde group of glyoxal and, secondly, the unreacted glycine acts as additional supplement to the cells.

Figure 1b shows the viability of L929 that were cultured with the extraction media that had been prepared from the neat and the cross-linked electrospun zein fiber mats for 7 d against that of the cells that were cultured with the fresh SFM. The two groups of the samples were also investigated here. Without the glycine treatment, the neat electrospun zein fiber mats still released no substance in the level that was toxic to the tested cells. Similarly, as the glyoxal content used to cross-link the fibrous membranes increased, the viability of the cells decreased from about 93% (for the fiber mats that had been cross-linked with 5 and 10% v/v glyoxal) to about 81% (for the fiber mats that had been cross-linked with 15% v/v glyoxal). Compared with the viability of the cells that were cultured with the

extraction medium that had been prepared from the fiber mats that were cross-linked with 15% v/v glyoxal for 3 d, that of the cells that were cultured with the extraction medium that had been prepared from the same materials for 7 d was unexpectedly greater. This could be a result of the change in the chemical structure of the toxic species that was released into the medium to the non-toxic one during prolonged incubation. For the glycine-treated samples, the viabilities of the cultured cells were considerably greater than those of the cells that had been cultured with the fresh medium and the extraction media

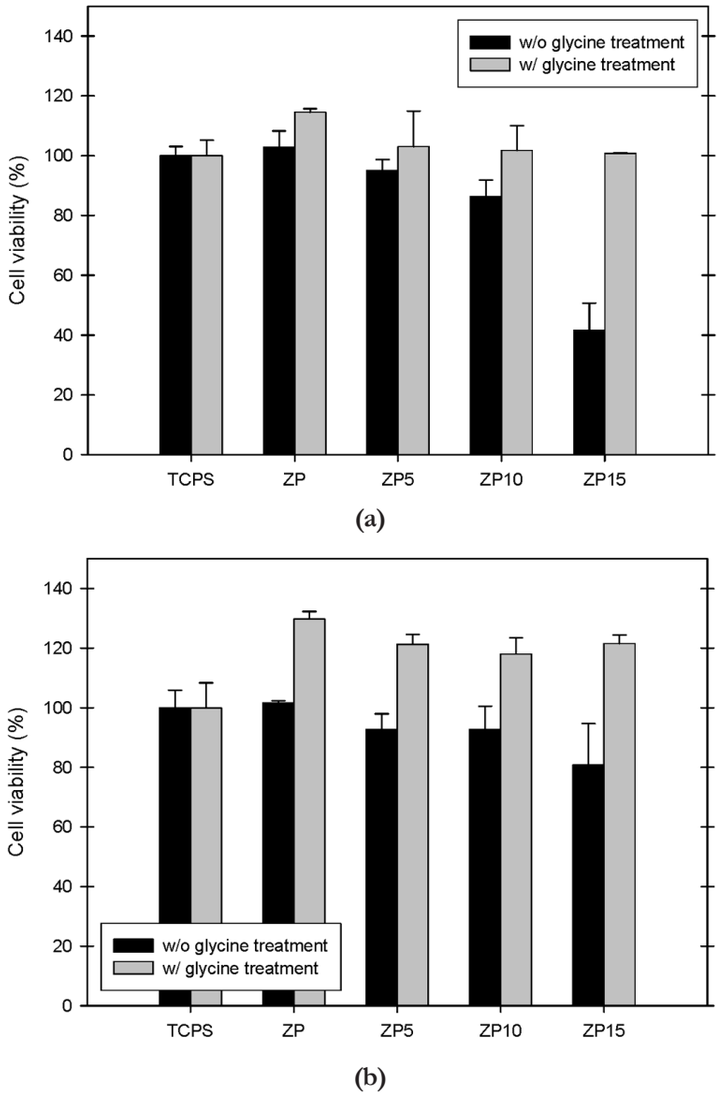
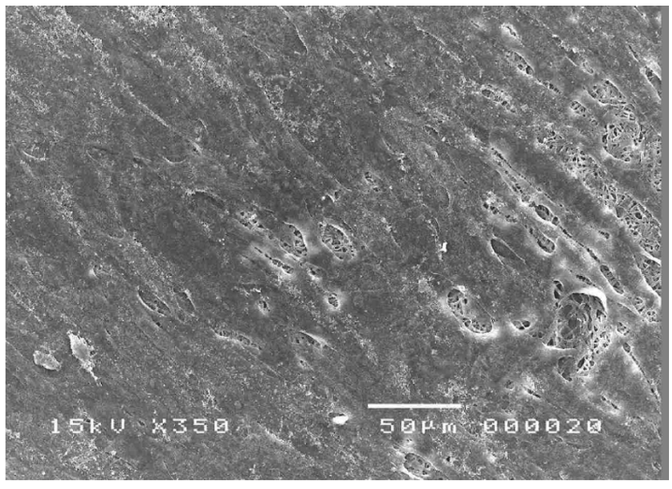


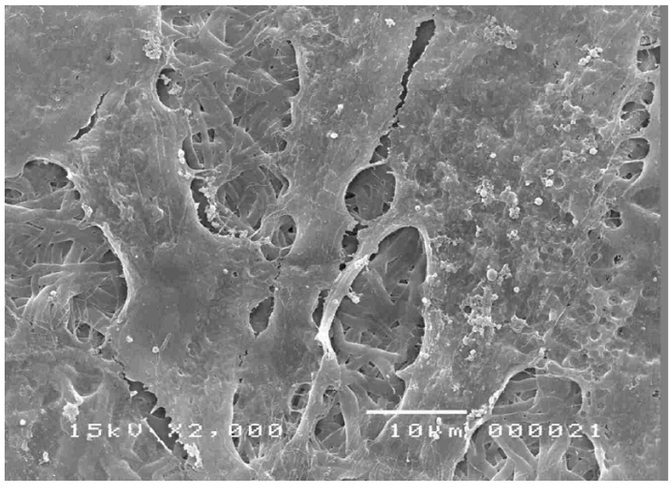
Figure 1. Viability of mouse fibroblasts (L929) that were cultured with extraction media prepared from the neat and the cross-linked electrospun zein fiber mats that had been submerged in fresh serum-free medium (SFM) for (a) 3 and (b) 5 d against that of the cells that were cultured with the fresh SFM (n = 3).

from the non-treated samples. This should be a result of the much greater amounts of glycine that had been released into the extraction media during the prolonged incubation, as the greater amounts of glycine helped to promote the growth of the cells [31], in addition to the blocking of the unreacted aldehyde group of glyoxal [32].

The potential for use of the cross-linked electrospun zein fiber mats as scaffolds for cell/tissue culture was evaluated with the fiber mats that had been cross-linked with 10% v/v glyoxal. Figure 2 shows representative SEM images at two magnifications of human foreskin fibroblast cells (HFF) that had been cultured on the substrates for 5 d. In the low magnification image (350x), it can be seen that a good number of cells attached well on the



(a)



(b)

Figure 2. Representative SEM images of human foreskin fibroblast cells (HFF) that had been cultured on the cross-linked electrospun zein fiber mats (i.e., ZP10) for 5 d at two magnifications of (a) 350x and (b) 2,000x.

surface of the cross-linked fibrous membranes. It is also seen that the cells, after 5 d of cell culturing, reached the confluence to form the hypothetical monolayer construct on the fibrous membrane surface. Interestingly, in the empty, uncovered space of the substrate surface, the fibrous structure was clearly seen and this confirmed the legitimacy of the cross-linking procedure utilized here. In the high magnification image (2,000x), the expansion of the cytoplasm of the attached cells was clearly observed. Based on the obtained results, it is confirmed that the chosen cross-linked electrospun zein fiber mats were non-toxic to the cells and could be used as substrates for cell/tissue culture.

4. CONCLUSION

Ultra-fine zein fibers were successfully prepared by electrospinning from zein solutions in 80% v/v ethanol aqueous solution. Various processing conditions (i.e., solution concentration, applied potential and collection distance) were investigated to obtain the optimal condition for electrospinning. Beaded fibers were the common features for the zein solutions with concentrations lower than 35% w/v. The diameters of the individual fiber segments were found to increase with an increase in either the solution concentration or the applied potential, while they decreased with an increase in the collection distance. An increase in the concentration of the zein solutions also resulted in less tendency for bead formation. In all of the spinning conditions investigated, the diameters of the obtained fibers ranged between 0.32 and 1.10 μm. Cross-linking of the electrospun zein fiber mats, in an attempt to preserve their fibrous structure upon submersion in an aqueous medium, was carried out by adding varying amounts of glyoxal aqueous solution (i.e., 0, 5, 10, and 15% v/v, based on the volume of the zein solution) to 40% w/v zein solution. Cross-linking improved the mechanical integrity of the materials and increasing the amount of glyoxal aqueous solution in the base zein solution resulted in observed increases in the mechanical properties, excepted for the cross-linked fiber mats from the solution that contained 15% v/v glyoxal aqueous solution that showed equivalent values to those of the neat materials. The improvement in the physical appearance of the cross-linked electrospun zein fiber mats was evident upon their submersion in distilled water at either room temperature (i.e., 25 ± 1°C) or 37°C for 1 or 5 d, as the underlying fibrous structure was still intact, despite the observation of the fusing and the flattening of the fibers. The potential for use of the cross-linked electrospun zein fiber mats as scaffolding materials for cell/tissue culture was assessed by the indirect cytotoxicity evaluation using mouse fibroblasts (L929) and the results showed that the toxicity level increased with an increase in the amount of glyoxal aqueous solution that was used to cross-link the materials. Notwithstanding, such the toxicity could be alleviated with the treatment with glycine. Finally, the zein fiber mats that had been cross-linked with 10% v/v glyoxal aqueous solution supported the growth of human foreskin fibroblasts (HFF) relatively well.

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