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Process parameters optimization for tissue engineered chitosan/gelatin nanofibrous scaffolds

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ABSTRACT

In this study, nanofibers were electrospun from chitosan and gelatin at several blends (chitosan/gelatin: 80/20, 60/40, 50/50, 40/60, 20/80) with different processing parameters (voltage, flow rate and distance between the tip of the needle and collector). Fourier transform infrared (FTIR), scanning electron microscopy (SEM), *In Vitro* degradation and tensile test were utilized to evaluate scaffolds for biomedical applications. The samples with 20% chitosan and 80% gelatin under special processing conditions (flow rate: 0.1 mL/h, voltage: 12 kV and distance: 16 cm) had the least amount of droplets and beads. Tensile mechanical test showed that the crosslinked nanofiber scaffold with 20% chitosan and 80% gelatin is the best choice when mechanical properties are required. In addition, the crosslinked scaffolds with 20% chitosan and 80% gelatin and 50% chitosan and 50% gelatin had the least and most amount of degradation respectively.

Keywords: *Nanomaterials, Electrospinning, Tissue engineering, Chitosan, Gelatin.*

1. INTRODUCTION

Recently, tissue engineering provided new medical therapies using polymeric biomaterials, which is a recently developed approach which plans to overcome the limitations of organ transplantation by providing man-made tissues and organs to patients extremely in need of them [1–4]. Although the final purpose of tissue engineering is to remanufacture lost human tissues, tissue engineered scaffolds as a disciplinary subject is applied in distinctive fields (e.g., drug delivery [5-7] and immunology [8]). The essential approach in tissue engineering involves the fabrication of scaffolds with cells to produce a functional tissue suitable for implantation. The main subject for tissue engineered scaffolds is to plan and manufacture biodegradable matrices that can imitate the componential and structural aspects of extracellular matrices (ECM) [4]. In human body and other living systems, extracellular matrix (ECM) plays a basic role in controlling cell behavior, while scaffold plays a basic role in tissue engineering. Nanofiberous scaffolds are applied as proper environment for cell attachment, and proliferation due to likeness to physical dimension of natural extracellular matrix [9-11]. For this reason, many methods have been developed and used to fabricate nanofibers such as self-assembly [12], dry and wet spinning [13], phase separation [14], electrospinning [15–16], etc. Among them, electrospinning has been one of the simple, effective and versatile processes to fabricate continuous nanofibers from kinds of polymers. A fundamental electrospinning system usually consists of three main components: a high voltage power supply, a spinneret and a grounded collecting plate (usually a metal screen, plate, or rotating mandrel). In this method, an electrical potential is applied between a droplet of a polymer solution held at the end of the nozzle and a grounded collector. When the applied electric field overcomes the surface tension of the droplet, a charged jet of polymer solution, which is controlled by the electric field, is ejected. The jet grows longer and thinner until it is collected on the collector plate as fibers. Electrospun nanofibers present number of attractive characteristics such as a very large surface to volume ratio and a high porosity with a small pore size [17, 18]. The properties of the nanofibers can be simply controlled by adjusting the various parameters such as electric field strength, flow rate, distance between the spinneret and the collecting plate and ambient parameters such as temperature and humidity [19, 20]. In the field of nanofiberous scaffolds, a large number of polymers have been examined to provide excellent environment for tissue engineering applications. Among them, natural polymers such as chitosan and gelatin do not cause foreign body response. Although they are difficult for electrospinning (due to their high viscosity and low solubility in general organic solvents), many studies have demonstrated using mentioned polymers for the fabrication of electrospun nanofibers could be useful [21-30]. Gelatin is a biopolymer obtained from collagen by controlled hydrolysis, which is the most plentiful structural proteins found in the animal connective tissues. It is biocompatible, biodegradable and commercially available at low cost [24, 31] and has good cell adhesion and proliferation [32]. This biopolymer can be used alone or as a blend component to provide nanofiberous environment for tissue engineering. Electrospinning of gelatin/water system is impossible. It has been electrospun from various solvents such as formic acid and acetic acid [33, 34]. Anyway, if a scaffold fabricated from a single biopolymer, cannot present all desired properties, but using two or more biopolymers, it could be serve a scaffold with the desired characteristics. Apart from the cell affinity, morphology, biodegradability and the physical properties can be tailored using mixing biopolymers in

electrospinning. Hence, electrospun scaffolds formed by combination of different biopolymers appropriate an interesting option. Mixing of biopolymers can provide a pathway for access to new tissue engineering scaffolds with unique properties as compared to homopolymer scaffolds. Chitosan, as another polymer, is a biocompatible and biodegradable biopolymer obtained from chitin that is structurally similar to glycosaminoglycans (GAGs) a main component of ECM [35, 36]. When in chitin, the degree of deacetylation reaches 50%, chitosan is capable to form in aqueous acid solutions [37]. Chitosan shows excellent cell adhesion, proliferation, and antimicrobial properties [37-39]. It has also been widely used as biomaterials in pharmaceutics, wound healing, tissue-engineering and drug-delivery applications [16, 38, 40-47]. Electrospun mixing of chitosan and gelatin could imitate the composition structure in ECM. Also the electrospinning ability of chitosan can be increased by mixing it with other biopolymers that have excellent fiber-forming ability. In this study, we aim to manufacture a chitosan-gelatin nanofiberous scaffold using electrospinning, which can be used as ideal scaffold for tissue engineering applications. This work is aimed at investigating the properties of chitosan-gelatin nanofiberous scaffolds by FTIR, SEM, *In Vitro* degradation and tensile tests. Therefore, chitosan and gelatin were dissolved in an acetic acid solution. Next, gelatin–chitosan nanofibers were prepared by electrospinning and procedure parameters (such as the applied electric field, the distance between the needle with collector and feed flow rate) and the optimum conditions of eletrospinning were examined. A crosslinking agent was used to stabilize nanofiberous scaffold. Then, morphology, composition, biodegradability and mechanical properties of the nanofiberous scaffold were evaluated. Our results showed that the electrospun gelatin–chitosan nanofiberous scaffold have great capability in tissue engineering applications.

2. EXPERIMENTAL SECTION

2.1. Materials. Acetic acid, glutaraldehyde (the cross-linking agent) and gelatin were obtained from Merck (Germany). Chitosan (degree of deacetylation 0.85, MW 110 kDa) and lysozyme from chicken egg whites were purchased from Sigma–Aldrich Chemical Company. Also phosphate buffered saline (PBS) was purchased from Gibco (Germany). All products were used without further purification.

2.2. Electrospinning. Gelatin 30% (w/v) and chitosan 3% (w/v) were dissolved in solutions (with 80% acetic acid and 20% deionized water). Then the solutions were blended at the ratios of 80:20, 60:40, 50:50, 40:60 and 20:80 at room temperature with stirring for a period of 20 h. Scaffolds were fabricated by electrospinning method. The blended biopolymers were fed into a 1 ml syringe. The diameter of nozzle was 0.1mm. Also voltage (12 to 24 KV), flow rate (0.1 to 1 mL/h) and distance between the tip of the needle and collector (8 to 24 cm) were changed for accessing to an ideal nanofiberous scaffold.

2.3. Crosslinking.

The crosslinking procedure was fulfilled by placing the chitosan–gelatin nanofiberous scaffolds in a sealed desiccator containing 10 ml of 25% glutaraldehyde aqueous solution in a Petri dish. The scaffolds were placed on a holed shelf in the desiccator and it were crosslinked in an atmosphere of water and glutaraldehyde vapor at room temperature for 48h. After crosslinking, the samples were exposed in the vacuum oven at room temperature.

2.4. Fourier transform infrared (FTIR).

Chemical analysis of scaffolds was carried out using FTIR spectroscopy with Nicolet Brucker IFS-48 FTIR spectrophotometer with a KRS-5 prism over a range of 400–4000 cm^{-1} wavenumber.

2.5. Scanning electron microscopy (SEM).

The characterization of the nanofiberous scaffolds were determined using scanning electron microscopy (SEM, KYKY EM-3200, China). For preparing of samples, a small section of the electrospun fiber was sputtered with a thin layer of gold prior to SEM observation. The scanning electron microscopy was performed at accelerating voltage of 24 kV after sputter coating with gold.

2.6. *In Vitro* degradation test.

Crosslinked electrospun mats were cut into 1cm × 1cm pieces and weighed for degradation test. *In Vitro* degradation of the composite scaffolds was evaluated in 2 ml PBS (pH 7.4) with lysozyme (with 10mg/L concentration) in 12-well tissue culture plate at 37 °C for 30 days. At different time points (3 days, 10 days and 30 days) samples were removed from plates and then washed three times with distilled water and dried at room temperature for 48 h. PBS was changed every 5 days. Weight loss of each sample was calculated as follow:

$$ML\ (\%) = [(W_0 - W_d) / W_0] \times 100$$

Where ML is mass loss, W_0 is initial weight of the sample and W_d is the weight of the degraded sample at different time points.

2.7. Tensile test.

The tensile testing of scaffolds (30×10 mm^2) was performed by a universal materials tester (H5 K-S, Hounsfield, UK) with a 50 N load cell at ambient temperature 20 °C and humidity 65%. The stretching rate of the measurement was 10 mm/min.

3. RESULTS SECTION

3.1. Fourier Transform Infrared (FTIR).

Different ratio of chitosan and gelatin in different electrospun scaffolds lead change in FTIR spectrum as seen in Figure 1(a). Results show that an absorption peak at 1680 cm^{-1} which represents the amide I characteristic band. Also –C=O groups in gelatin is capable for forming hydrogen bonds with –OH and –NH_2 groups in chitosan. This is resulting in the increased NOH bending and the decreased COO stretching vibration in both of

gelatin and chitosan at 1148 cm^{-1} and 1060 cm^{-1} [48]. Furthermore, it is possible to form ionic bonds between chitosan and gelatin. These molecules have a capacity for forming a complex with oppositely charged ionic polymers (the anionic –COOH group in gelatin and cationic polysaccharide in chitosan). Therefore, these interactions form a polyanionic–polycationic complex [49]. The FTIR spectrum of electrospun and crosslinked chitosan and gelatin complex (50:50) in Figure 1(b) indicated that peaks at about 1541 and 1654 cm^{-1} were presented. Because of crosslinking reactions between aldehyde groups of glutaraldehyde and amino groups of gelatin and chitosan, these peaks were appeared. As a result of the cross-linking reaction, nanofiberous mats were yellow after cross-linking while the non-crosslinked electrospun mats were visibly white. Additionally the absorption peak of the free amino group and OH group shifted from 3278 to 3420 cm^{-1}. Once cross-linking, the C–O–C–O–C structure was formed.

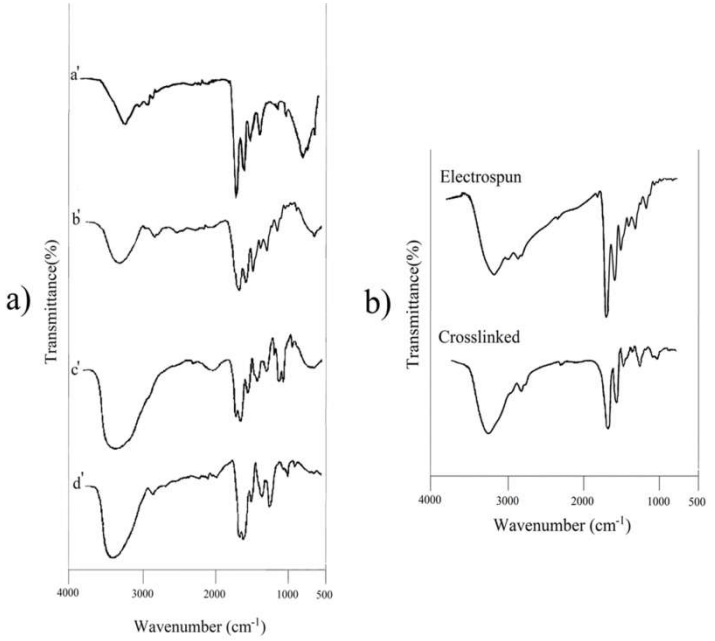

Figure 1. (a) FTIR spectrum of electrospun chitosan and gelatin scaffolds. Chitosan/gelatin ratio: (a') 80:20, (b') 60:40, (c') 40:60 and (d') 20:80. (b) FTIR spectrum of electrospun and crosslinked chitosan and gelatin complex (50:50).

3.2. Electrospinning.

Figure.2 showed SEM micrographs of the electrospun chitosan and gelatin complex nanofibers (flow rate: 0.1 mL/h, voltage: 12 kV and distance: 16 cm). Fibers were electrospun from chitosan and gelatin blend fibers with volume ratios of chitosan/gelatin 80:20, 60:40, 50:50, 40:60 and 20:80. This figure indicated that the morphology of fabricated nanofibers affected by the ratio of chitosan and gelatin. As seen in the Figure.2 (a) and (b), when the ratio of chitosan higher than gelatin in scaffold, maximum droplets, beads and defects were visible. By increasing the gelatin portion in scaffolds, when the ratio of gelatin and chitosan were exactly equal, homogeneous fibers with lower amount of beads and droplets were formed (Figure.2 (c)). Also when this ratio was up to 80:20, homogenous nanofibers of chitosan–gelatin blend were formed as also seen in (Figure 2(e)). This sample had minimum beads and droplets. Thus any further reduction in the ratio of gelatin induced significant bead formation. Therefore gelatin could improve the ability of chitosan fiber-forming, and the produced nanofibers became smoother. For manufacturing of beadfree and smooth fibers, as an ideal scaffold, different parameters such as applied voltage, distance between nozzle and collector and flow rate of solution were varied.

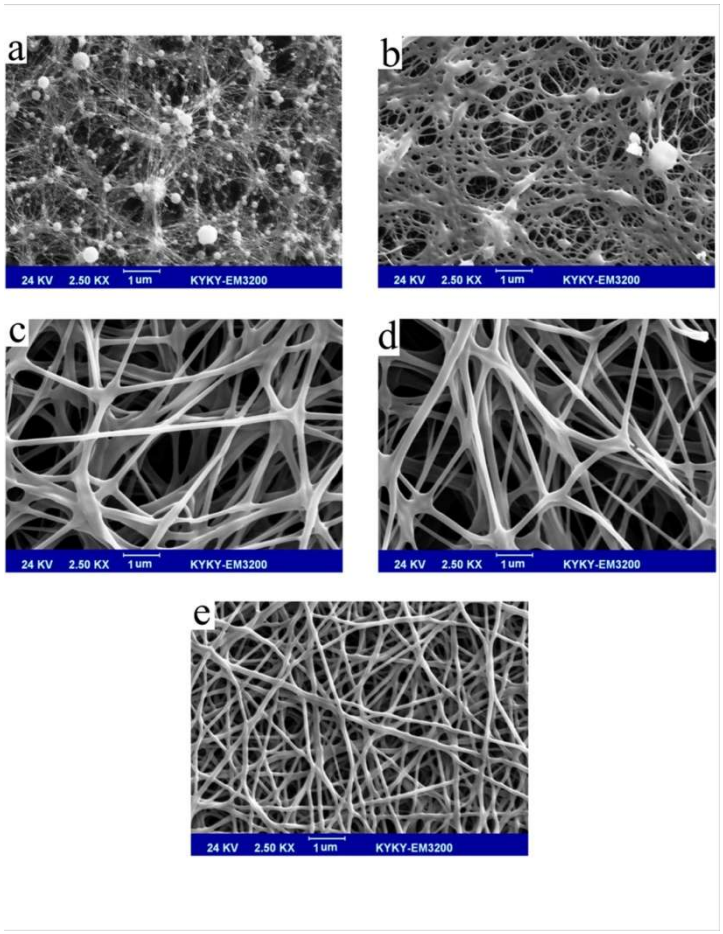

Figure 2. Scanning electron micrographs (a, b, c, d and e) of electrospun chitosan and gelatin complex nanofibers. Chitosan/gelatin ratio: (a) 80:20, (b) 60:40, (c) 50:50, (d) 40:60 and (e) 20:80.

It has been showed that the fiber diameters of the electrospun nanofiber depend on the mentioned parameters. First, the distance between nozzle and the collector was examined. In this examination, distance was varied from 8 to 24 cm as seen in Figure 3 and 4 (The ratio of chitosan /gelatin, voltage and flow rate were 80:20, 12kV and 0.1 mL/h respectively in all experiments).

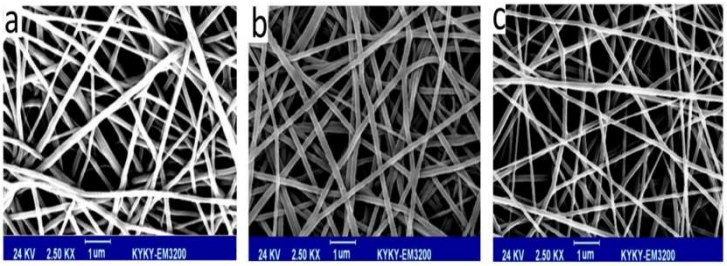

Figure 3. Scanning electron micrographs (a, b and c) of electrospun chitosan and gelatin complex nanofibers in different distance between nozzle and collector: (a) 8cm, (b) 16cm and (c) 24cm.

By increasing the distance, despite there was not any difference in alignment, amount of bead and distribution of fibers (Figure 3), the fiber diameters (Figure 4) were decreased from 200±40 to 160±20 nm.

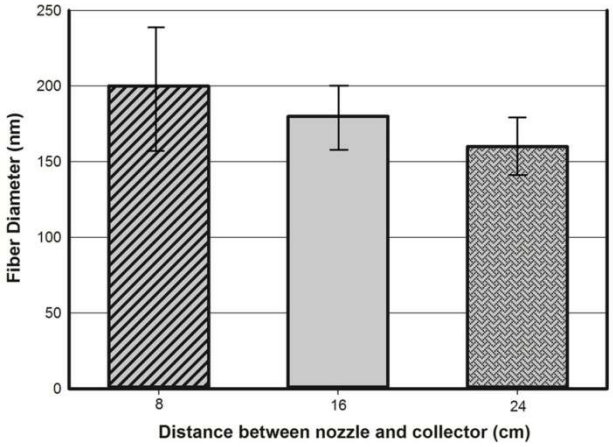

Figure 4. The effect of distance between nozzle and collector on size of electrospun chitosan and gelatin fibers. Electrospun fibers were examined by SEM and data are expressed as means ± SD, n=10.

Then the flow rate was increased from 0.1 to 1 mL/h (Figure 5 and 6). The ratio of chitosan /gelatin, voltage and distance were 80:20, 12kV and 16cm respectively in all experiments. It was showed that both of morphology (Figure 5) and fiber diameter (Figure 6) were affected by the variation of flow rate. When flow rate was increased, the amount of beads was increased but the range of fiber diameters decreased from 220±30 to 110±10. Regarding visible beads, sometimes, in the higher amount of the flow rate, we expected that more solution was exited. For this condition, because of the challenge between spreading out the polymer jet into electric field and leaving of solution, solution does not have any chance to get charged and would be changed as droplets and formation of beads and defects.

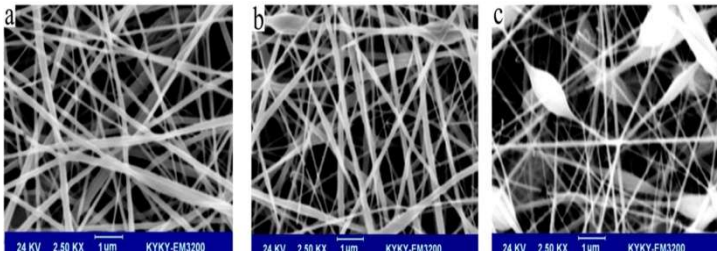

Figure 5. Scanning electron micrographs (a, b and c) of electrospun chitosan and gelatin complex nanofibers in different flow rate: (a) 0.1 mL/h, (b) 0.5 mL/h and (c) 1.0mL/h.

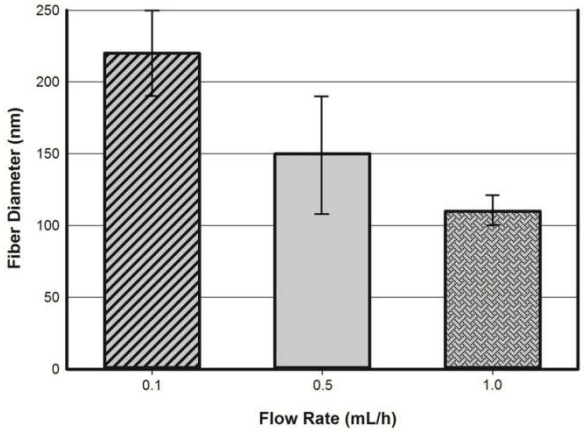

Figure 6. The effect of flow rate on size of electrospun chitosan and gelatin fibers. Electrospun fibers were examined by SEM and data are expressed as means ± SD, n=10.

Figure 7 and 8 are demonstrated that the effect of applied volatage on fiber morphology was studied. The volatage was varied from 12 to 24 kV. The ratio of chitosan /gelatin, distance and flow rate were fixed 80:20, 16cm and 0.1 mL/h respectively in all experiments. Results showed that fiber morphology dependents on variation of applied voltage. These effects exactly were similar effects of variation of flow rate on morphology. When the applied voltage changed from 12kV to 24 kV beads and droplet was started to form according to Figure 7.

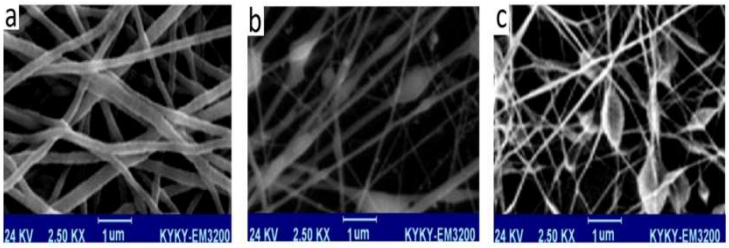

Figure 7. Scanning electron micrographs (a, b and c) of electrospun chitosan and gelatin complex nanofibers in different voltage: (a) 12kV, (b) 18kV and (c) 24kV.

Furthermore, fiber diameter decreased from 190±20 to 120±30 (Figure 8). The appearance of beads and droplets is because of increasing of force and instability of charged jet produced by the stronger electric field [28].

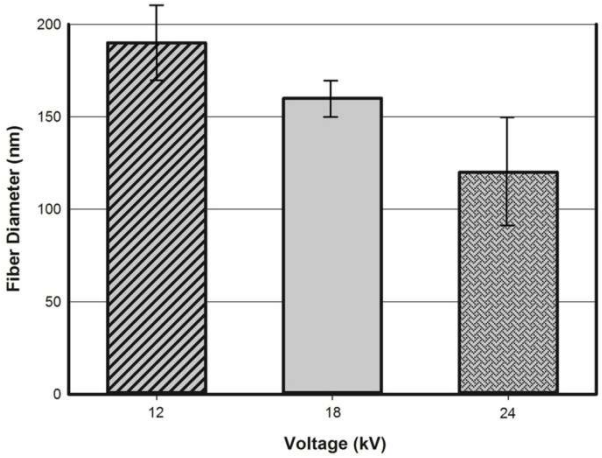

Figure 8. The effect of voltage on size of electrospun chitosan and gelatin fibers. Electrospun fibers were examined by SEM and data are expressed as means±SD, n=10.

3.3. *In Vitro* degradation test.

Analysis of mass loss of crosslinked chitosan and gelatin electrospun scaffold is showed in Figure 9. After couple of days of incubation period in PBS and lysozyme, fibers of chitosan and gelatin scaffolds started to degrade and by increasing the incubation period, each other was increased. After 3 days of incubation period, all the scaffolds indicated significant mass loss. Also In first time points, samples with the ratio of 50:50 had the most degradation in comparison with other samples and the same pattern is continued up to the end of experiments. In all time points, samples with the ratio of 20:80 (chitosan : gelatin) had the least degradation and by increasing ratio of chitosan to 40% and 50%, mass loss was increased too. But in the higher of chitosan ratio (60% and 80%), the amount of degradation almost had a decreased route. These variations may be due to the variation in the density of hydrophilic groups in structure. According to these results, the speed of degradation between 3rd day and 10th day was the maximum. The degradation speed in after 10th day was decreased. This reduction is because of the saturation of lysozyme transforming and also the shortage of acetyl groups for banding to enzyme.

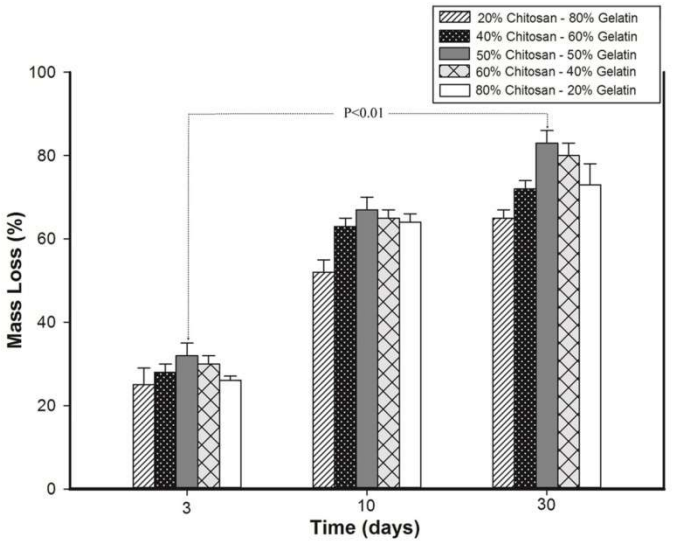

Figure 9. *In Vitro* degradation of scaffolds with different ratio of chitosan and gelatin for various length of incubation (3, 10 and 30 days).

3.4. Tensile test.

The mechanical properties of a scaffold for tissue engineering applications are very important. Tensile testing was directed to evaluate how the mechanical properties of the nanofiberous scaffolds were influenced when scaffolds had the different component of chitosan and gelatin. Also this test assessed the effects of crosslinking procedure on mechanical properties of mats. Figure 10 demonstrated the tensile strength and elongation of electrospun chitosan and gelatin nanofiberous scaffolds. Electrospun mats with 80% gelatin (sample e') had the maximum tensile strength and tensile elongation, and the average of these amounts decreased with the increase in chitosan content in the fibers, whereas the weakest tensile strength and elongation appeared in sample a'. This results confirmed again that the mechanical properties of chitosan is weak and for tissue engineering applications, whereas mechanical properties is required, chitosan could not be a appropriate option alone and it need to be added to other biomaterials that have many better mechanical properties. Additionally, as seen in the Figure 10(a) and (b), crosslinking agent (glutaraldehyde vapor) had a positive effect on tensile strength and a negative influence on tensile elongation at break. Fibers were bonded with each other after crosslinking. It could restrict the slippage. Thus the tensile strength was dramatically enhanced. According to Figure 10(a), crosslinked scaffolds had significantly higher amount of tensile strength (200% to 300% more) in comparison with non-crosslinked scaffolds. On the other hand, cross-linked chitosan and gelatin mats indicated brittle mechanical behavior.

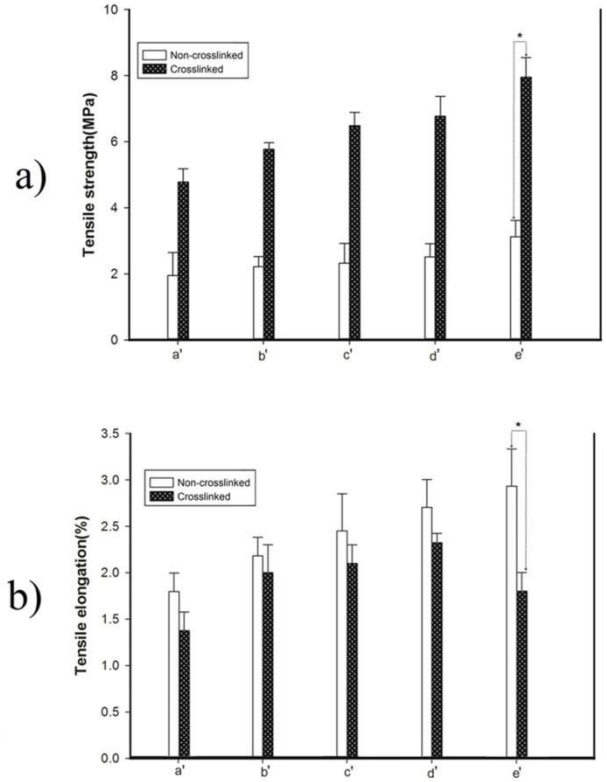

Figure10. (a) Tensile strength and (b) tensile elongation of (a', b', c', d' and e') of chitosan and gelatin complex scaffolds. Chitosan/gelatin ratio: (a') 80:20, (b') 60:40, (c') 50:50, (d') 40:60 and (e') 20:80. * P < 0.005.

4. CONCLUSIONS

In this work, we fabricated crosslinked electrospun chitosan and gelatin nanofibers with different weight ratio to mimic natural ECM for tissue engineering applications. Different parameters such as different ratio of chitosan and gelatin, Distance between nozzle and collector, flow rate, applied voltage, selected to examine their effects on morphology of mats. Then fourier transform infrared (FTIR), scanning electron microscopy (SEM), *In Vitro* degradation test and tensile test were utilized to study the properties for tissue engineering application. Results showed that the chitosan/gelatin mats with ratio of 80:20 under special processing conditions (flow rate: 0.1 mL/h, voltage: 12 kV and distance: 16 cm) had the least amount of droplets and beads in comparison with other samples. Besides, tensile test showed that the crosslinked nanofiber scaffold with 20% chitosan and 80% gelatin is the best choice when mechanical properties are required. Also for biological environments applications, the crosslinked scaffold composed of chitosan and gelatin with ratio of 20:80 and 50:50 had the least and most amount of degradation respectively. Further studies will be focused on cell culture and In Vivo study to complete evaluation of biocompatibility.

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