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Electrospinning and characterization of konjac glucomannan/chitosan nanofibrous scaffolds favoring the growth of bone mesenchymal stem cells

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ABSTRACT

Many natural polymers could not be electrospun from their aqueous solutions due to lack of organic solvents. In this study, konjac glucomannan (KGM) scaffolds with the average fiber diameter ranging from 150 nm to 350 nm were fabricated by electrospinning its aqueous solution. The stability of KGM based scaffolds was improved after alkali treatment at a low concentration without any other chemical crosslinking agents involved. Meanwhile, KGM/chitosan bicomponent nonwoven membranes were also easily obtained from their dilute acidic solution, and the average fiber diameter decreased from 350 nm to 180 nm with the increase in chitosan content. The study on the biological properties shows that the nanofibrous scaffolds provide more suitable space room for bone mesenchymal stem cells to adhere than the bulk films. Moreover, the addition of KGM improves the biocompatibility of chitosan materials.

1. Introduction

Electrospinning is a simple technique to fabricate three-dimensional scaffold with many advantages including high surface-to-volume ratio, high porosity, flexibility, light weight, etc. (Agarwal, Wendorff, & Greiner, 2008; Greiner & Wendorff, 2007). In the past, a broad range of polymers has been processed by electrospinning to architect the tissue engineering scaffold (Huang, Zhang, Kotakic, & Ramakrishna, 2003). Synthetic biodegradable polymers, such as poly (ε-caprolactone) (PCL), poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and the copolymer poly (lactide-co-glycolide) (PLGA) are examples for the case where organic solvents are used for spinning (Cui et al., 2006; Hong et al., 2008; Lee, Kim, Khil, Ra, & Lee, 2003; Park, Kang, Lee, Min, & Park, 2006). For biomaterials, natural polymers are generally favored over synthetic polymers with the fact that they have better cytocompatibility. Therefore, fabrication of nanofibrous scaffolds of natural polymers has attracted much attention (Dror et al., 2008; Nie et al., 2008, 2010; Um, Fang, Hsiao, Okamoto, & Chu, 2004). It is well known that water is often a good solvent for natural polymers, thus, electrospinning their aqueous should be an ideal choice from the reasons of environmental protection, economic cost, the health of the operator and biomedical applications. However, for most natural polymers, water-based electrospinning is often difficult to perform due to lack of suitable organic solvents (Li et al., 2006; Nie et al., 2008). In addition, a major drawback of water-soluble fibrous scaffolds would be the need of chemical crosslinking to prevent rapid hydrolysis of the delicate fibers in aqueous media (Newton et al., 2009; Schiffman & Schauer, 2007; Sisson, Zhang, Farach-Carson, Chase, & Rabolt, 2009; Xu et al., 2009). However, the commonly used crosslinking agents are often cytotoxic agents such as glutaraldehyde.

Konjac glucomannan (KGM) is a neutral polysaccharide derived from the tuber of *Amorphophallus konjac* C. Koch. KGM consists of α-glucose and α-mannose linked by β-1,4-linkage. It has mannose and glucose units in a molar ratio of 1.6:1.0, and the presence of some branching points at the C-3 position of the mannoses is suggested. An acetyl group is attached to 1 per 19 sugar residues (Huang, Takahashi, Kobayashi, Kawase, & Nishinari, 2002). It is widely accepted that the presence of this group confers solubility on the konjac glucomannan in aqueous solution. Maekaji reported that the molecules of KGM, which lost their acetyl groups with the aid of weak alkalis, aggregate in part with one another through a linkage such as the hydrogen bond, by which the molecules come into a network structure (Maekaji, 1978). That is, the stability of KGM materials in water improved without the treatment of toxic chemical reagents. KGM can be extruded into films or blend membranes, which can be used in many fields, such as food, chemical engineering and biomaterials (Wang et al., 2008; Zhang, Xie, & Gan, 2005). However, to date, there was no report to refer the

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preparation of KGM nanofibrous scaffolds and their biomedical applications.

Chitosan has been proven to be an ideal biopolymer with a wide variety of biomedical and industrial applications (Muzzarelli, 2009, 2011; Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). However, its nanofibrous scaffolds are often fabricated by electrospinning chitosan concentrated acid solution or toxic fluorinated compounds. Therefore, a wide fabrication of chitosan nanofibrous scaffold may be hindered.

In this study, nanofibrous scaffold of KGM was prepared by electrospinning its aqueous solution, and KGM was blended with chitosan to aid the fabrication of the bicomponent nanofibers from a dilute acid solution. The stability of nanofibrous membrane was investigated after alkali treatment. The cell adhesion on KGM nanofibrous scaffold and the composite scaffold of chitosan and KGM was evaluated by bone marrow mononuclear cell culture experiment.

2. Materials and methods

2.1. Materials

Konjac glucomannan (KGM, intrinsic viscosity at 25 °C, ~30,000 MPa s) was purchased from Qingjiang Konjac Co. (Wuhan, China). Chitosan (Mw 200 kDa) was purchased from Fluka. Ethanol and NaOH were obtained from Beijing Chem. Co. (Beijing, China). All the reagents were used as received.

2.2. Methods

2.2.1. Preparation of spinning solution

KGM solutions were prepared in water with the concentrations of 0.6% (w/v, w in gram and v in milliliter), 0.9% (w/v), 1.1% (w/v) and 1.5% (w/v), respectively. KGM/chitosan blend solutions were prepared as follows: in order to make the matrix dissolve, all solvent used in this blend solutions was the mixture of double distilled water (d) and acetic acid (a) (volume ratio of water to acetic acid = 9/1). A precise amount of KGM and chitosan was dissolved in above mixed solvents under gentle stirring for 2 h. Finally, mixed solutions with different KGM/chitosan weight ratios (60/15, 45/30 and 50/25) and constant total concentration (1.5%, w/v) were prepared.

2.2.2. Electrospinning

The temperatures of all electrospinning solutions, spinneret and the environment were controlled at 40 ± 3 °C, while the relative humidity of the electrospinning environment was also considered and controlled at around 30%. The electrospinning solution was placed into a 5 ml syringe with a capillary tip with an inner diameter of 0.3 mm. A syringe pump was used to feed the polymer solution and the feeding rate was fixed at 50 μl/min. A DC high voltage generator (The Beijing Machinery & Electricity Institute, China) was applied to produce voltages ranging from 0 to 50 kV. The applied voltage was fixed at 22 kV in this experiment and the tip-to-collector distance was fixed at 8 cm. The fibers produced were put into the vacuum oven at 50 °C for overnight to dry off residue solvents.

2.2.3. Hydrolysis of KGM based nanofibers

The hydrolysis reaction was carried out in 5 ml blend medium (volume ratio of ethanol to water is 8/2). The KGM and KGM/chitosan fibrous membranes (~20 μm in thickness, 1 cm × 2 cm in size) were immersed into the blend medium containing 0.025 M of NaOH at 4 °C and kept for different time intervals to fulfill the hydrolysis reactions, respectively. After hydrolysis, the KGM and KGM/chitosan membranes were washed with water and ethanol for three times, respectively, and then were dried in vacuum at room temperature for three days to remove the residual solvents.

2.2.4. In vitro degradation of KGM fibrous membranes in PBS

The degradation experiments were carried out in PBS at 37 °C. Alkali treated membranes were immersed in PBS for different time intervals and then the samples were put into the vacuum oven at 50 °C for three days after washed with plenty of water. Finally, the remainder weight was weighed to evaluate the stability of KGM membranes besides the morphology changes.

2.2.5. Bone marrow mononuclear cell culture

The human bone marrow stem cells (MSCs) were supplied by the Institute of Urology Surgery of The First Affiliated Hospital of Nanchang University. Before cell seeding, the scaffolds were sterilized with 75 vol% ethanol (Damao Chemical Regent Factory of Tianjin) for 1 h and then incubated in PBS for 24 h to exchange ethanol. The MSC suspensions (5×10^6 cells/ml) were dispersed into the scaffolds by injection using a syringe. The cell-containing scaffolds were then incubated at 37 °C in a 5% CO_2 incubator for 1 day. The cells seeded in all the specimens were obtained from the same donor.

2.2.6. Characterization

The electron microscope (JEOL JSM-6700F, Japan) was used at the accelerating voltage of 5 kV. Each sample was sputter-coated with gold for analysis. Infrared spectra were recorded on an infrared spectrometer (IR, BRUKER TENSOR 27, Germany) at 4 cm^{-1} resolution with 32 scans in the range of 4000–400 cm^{-1}. Cells were observed by a fluorescence microscope (Leica DMI6000B). After culturing for 1 day, the scaffolds with MSCs were stochastically isolated and observed under the immunofluorescence microscope. The karyons exhibiting a blue fluorescence were recognized to be MSC tagged by DAPI.

3. Results and discussion

3.1. Preparation of nanofibrous scaffold

KGM nanofibers were fabricated by electrospinning its aqueous solution. The environmental temperature and KGM aqueous solution temperature were controlled at 40 °C to make the solvent system evaporate faster. At first, 0.6% (w/v) KGM aqueous solution was selected for electrospinning. We found that the jet was stable and an ultra-thin fibrous morphology was obtained even at a low concentration as shown in Fig. 1(A). Thereafter, KGM aqueous solutions with increased concentration were used for electrospinning. It can be seen from Fig. 1 that the average fiber diameter increased from 150 nm to 350 nm with the increase in polymer concentration.

For both in vitro and in vivo applications, materials are expected to maintain their structural integrity in aqueous medium. However, for most natural polymer, the morphology of fibrous membranes disappears rapidly in PBS due to their fast dissolution. Therefore, they must be crosslinked to achieve water-resistance for many applications. Herein, to achieve its availability, KGM membranes were treated with NaOH at a low concentration. Fig. 2 shows the FTIR spectra of KGM before and after alkali treatment. It can be seen that the characteristic absorption bands of the mannose in the KGM appeared at 804 and 885 cm^{-1}. The stretching peak of the C–H of methyl at 2908 and 2881 cm^{-1} and of the C–O–C at 1031 and 1069 cm^{-1} is assigned in KGM. It is evident that the hydroxyl stretching band around 3381 cm^{-1} becomes stronger due to the deacetylation. At the same time, the carbonyl stretching band at 1734 cm^{-1} disappeared with the increase of advanced reaction. Concomitant with the decrease of the carbonyl stretching band, the C–C(=O)–O stretching band at 1250 cm^{-1} characteristic

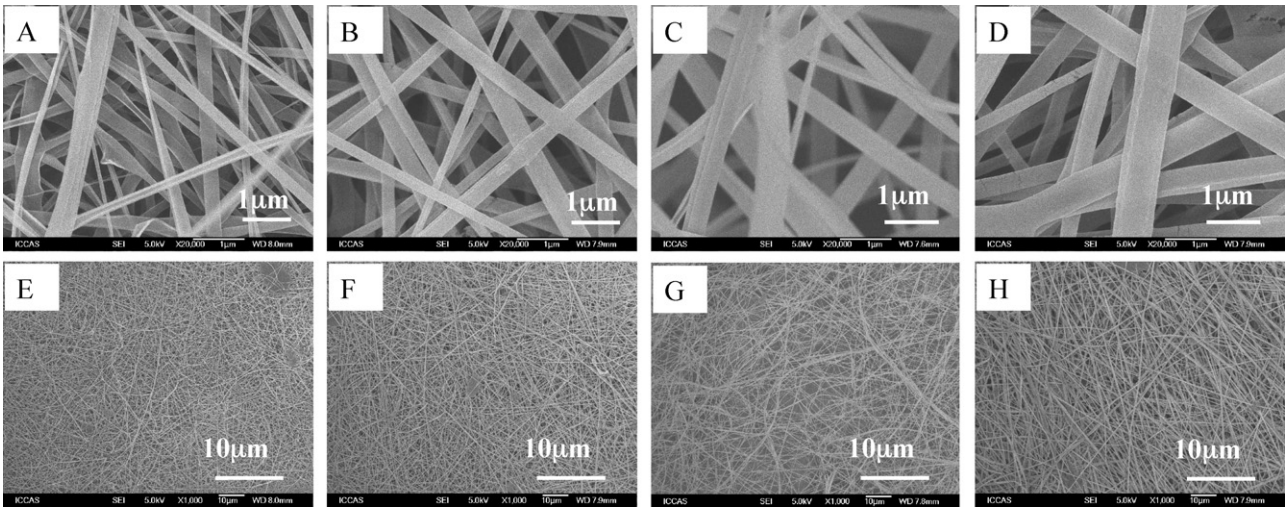

Fig. 1. SEM of KGM fibers electrospun from KGM solution with different concentrations: (A and E) 0.6% (w/v); (B and F) 0.9% (w/v); (C and G) 1.2% (w/v); (D and H) 1.5% (w/v).

of acetates also becomes smaller. This provides further evidence for the decrease of the acetyl groups.

Fig. 3 shows the evaluation of the stability of KGM fibrous membrane after alkali treatment for different times. It was found that all of the KGM membranes after alkali treatment still possessed nearly 100% of their initial weight after 30-day degradations in PBS. However, for original KGM fibers, the weight loss increased sharply after 1 day (Fig. 3A). In the experiment, the area of KGM fibrous membranes decreased rapidly, and its fibrous structure disappeared as seen in Fig. 3B. Although the alkali treated membranes of KGM became transparent after immersion in PBS due to the swelling of KGM fibers, the morphology was maintained very well and little adhesion was observed (Fig. 3C). These results indicated that KGM based fibrous membranes could achieve excellent stability via a safe and low cost method.

Nanofibers of KGM and chitosan were prepared. Fixed the overall concentration of the blend solution at 1.5% (w/v), the mass ratio of KGM and chitosan was changed. A stable jet was observed and smooth ultrathin fibers were collected during the spinning process. It can be seen that the smooth blend fibers with narrow distribution of fiber diameter have been obtained and the average diameter decreases from 350 nm to 180 nm with the increasing content of chitosan (Fig. 4).

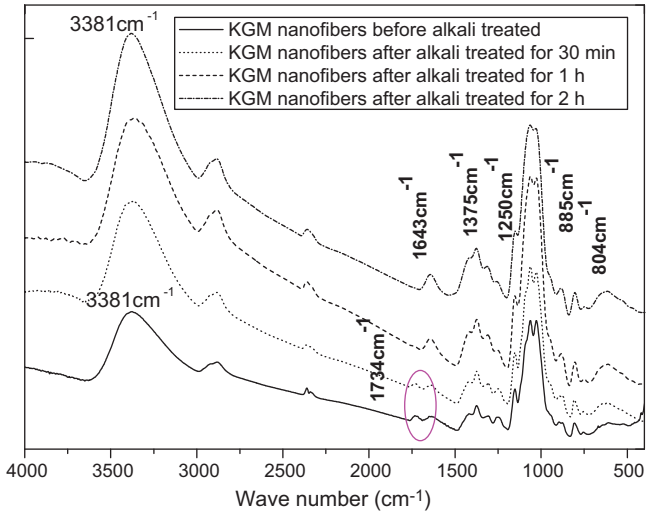

Fig. 2. FT-IR spectra of KGM fibers after alkali treatment.

The FTIR spectra of KGM/chitosan composite membranes are shown in Fig. 5. The absorption coupled band at 3404 cm^{-1} and 3174 cm^{-1} was assigned to the stretching of N–H groups and –OH in chitosan; the peaks at 1552 and 1265 cm^{-1} are assigned to the characteristic bending absorption band of amino group and the stretching of acrylamide III; the peaks at 1093 cm^{-1} were assigned to the characteristic absorption band of C6–OH. For KGM/chitosan blend films, some peaks disappeared or became weak due to interaction or superposition between groups of chitosan and KGM. The stretching of carbonyl at 1723 cm^{-1} of konjac glucomannan weakened and even disappeared; the absorption band around 3440 cm^{-1} shifted to a lower wave number with the increase of konjac glucomannan, indicating the gradual increase of intermolecular hydrogen bonds between chitosan and konjac glucomannan, and the stretching of intramolecular hydrogen bonds at 1643 cm^{-1} in konjac glucomannan coupled and shifted to a lower wave number, suggesting that the new hydrogen bonds occurred between chitosan and KGM in the blend film.

3.2. Cell culture and cell adhesion study

Biocompatibility and cell adhesion of KGM and KGM/chitosan fibrous membranes were evaluated by culturing with bone mesenchymal stem cells.

Fig. 6 shows fluorescence images of bone mesenchymal stem cells cultured on the glass substrate and the KGM fibrous membranes for only 1 day. The nuclei of the cells were stained blue. It can be seen that all cells appeared to adhere on the KGM nanofibrous membranes and exhibit normal morphology. However, there were fewer cells adhered on the glass substrate. The bone mesenchymal stem cells on glass substrate showed round morphology. It seemed that the bone mesenchymal stem cells do more favor the KGM fibrous membrane than adhere to themselves on glass substrate. These results indicated that the KGM fibrous membrane shows a desirable cell cytocompatibility.

Based on above-mentioned results, the cell affinity with different compositions of materials and morphologies of matrix was further investigated. Bone mesenchymal stem cells were cultured on different membranes, and the nuclei of the cells were stained blue. Fig. 7 shows the fluorescence images of stem cells cultured on KGM fibrous membranes and their composite membranes after 1 day. It is obvious that all of the matrix show desirable cell affinity, and the cells tend to adhere to these natural substrates. However, different cell densities on two types of surfaces became notable after 1-day culture, and the cell density on the fibrous mem-

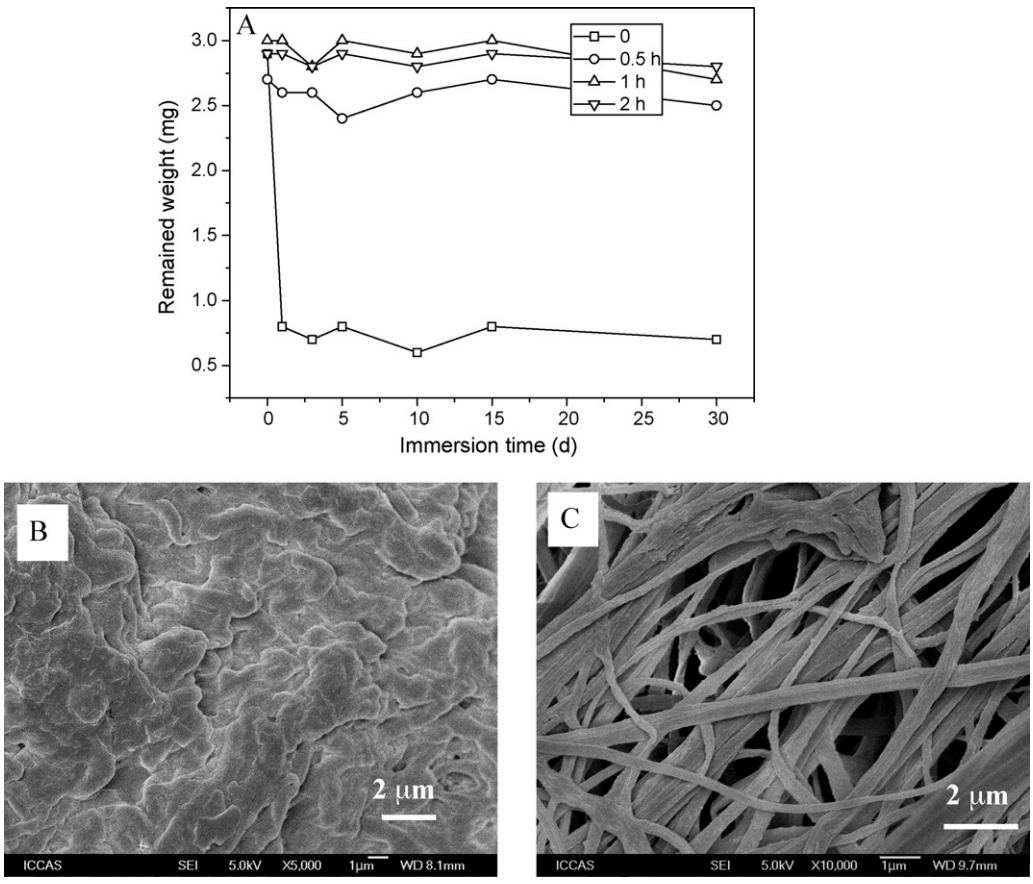

Fig. 3. Evaluation of water-resistance of KGM electrospun membranes after alkali treatment: (A) weight loss of alkali treated KGM fibrous membranes in PBS; SEM of (B) Pristine KGM membranes and (C) KGM membranes after alkali treatment for 30 min, respectively, immersed in PBS for 15 days.

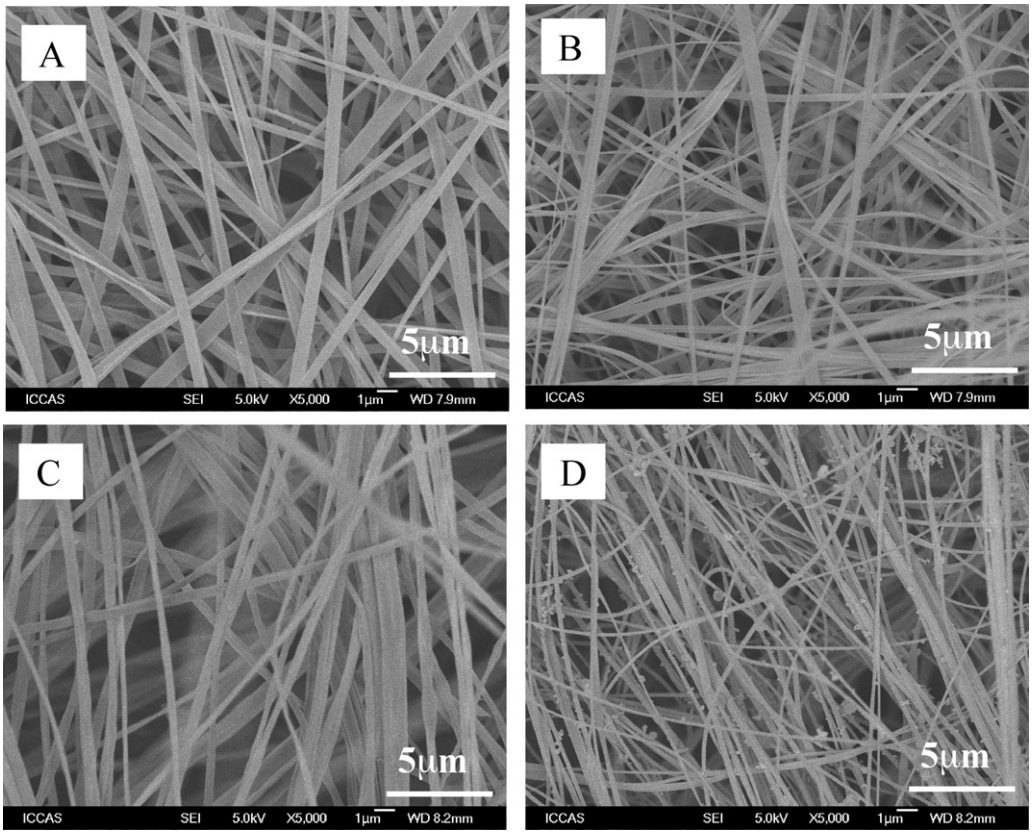

Fig. 4. SEM of KGM/chitosan blend fibers with different KGM contents: (A) 100%, (B) 80%, (C) 60%, and (D) 33%.

brane was higher than that on the casted film after 1-day culture. Meanwhile, the KGM based fibrous membranes present better cell viability than the chitosan membranes. This phenomenon is possibly related to the intrinsic nature of chitosan reported in previous literature (Chatelet, Damour, & Domard, 2001). Although chitosan possesses good biological compatibility and nontoxicity, chitosan is not suitable for cell attachment and proliferation due to the high density of amino groups. However, this feature of chitosan materials can be changed by introducing the other natural biopolymers, such as the blend materials of KGM/chitosan.

The surfaces of the KGM, chitosan and KGM/chitosan composite films should have different effects on cytotoxicity. It can be seen from Fig. 8 that only a fraction of cells could attach on the surface of chitosan matrix with the clusters. In contrast, when the materials were blended by KGM, they became substrates with properties much like those of controls. A lot of cells attached and spread on the KGM/chitosan blend membrane surfaces without apparent impairment of cell morphology. It was confirmed that the KGM/chitosan blend membranes had good cell affinity. Meanwhile, the fiber surface was completely covered by cells, and the cells on the fibrous surface (Figs. 8D and E) presented a typical polygonal morphology with extended filopodia compared to the casted films, where cells favor to gather together (Figs. 8A and B). The results may be due to that high surface to volume ratio of the nanofiber provides more room for the cell attachment than the bulk film. The dimensions

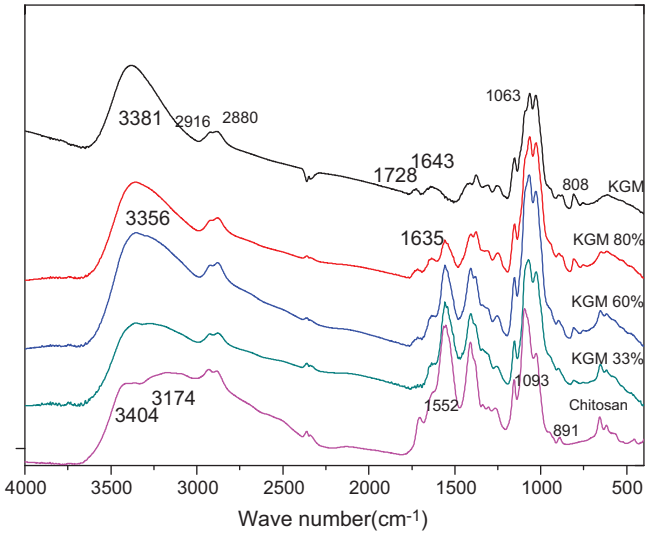

Fig. 5. FT-IR spectra of KGM/chitosan composite fibrous membranes.

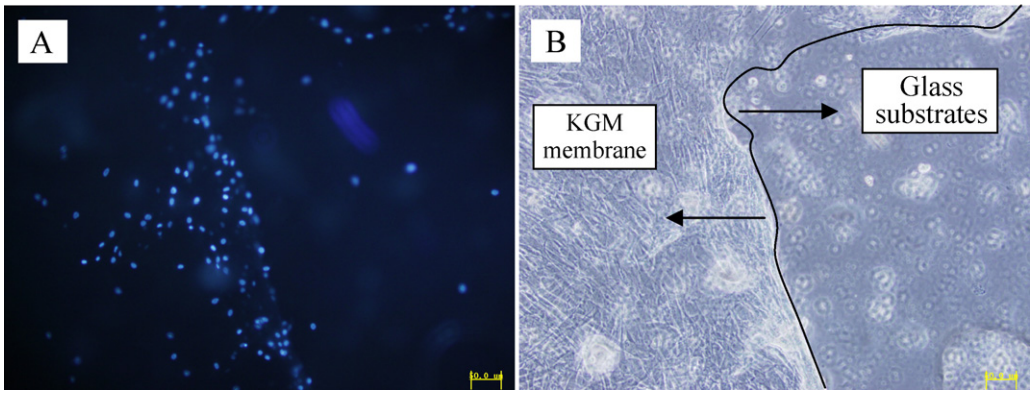

Fig. 6. Micro-images of stem cells cultured on KGM fibrous membranes and glass substrates after 1 day: (A) fluorescence graphs and (B) photographs.

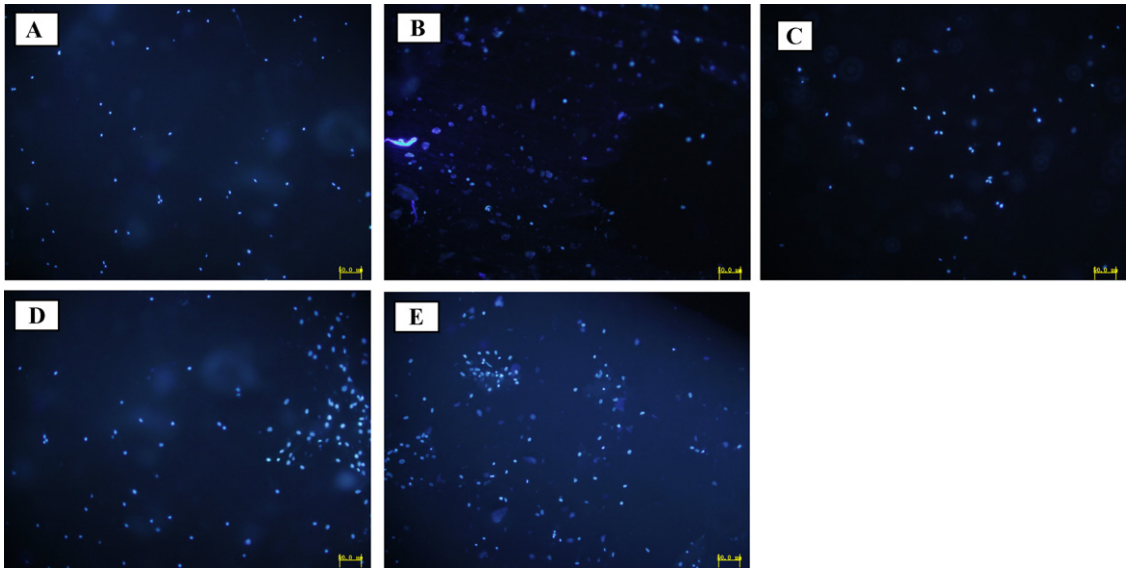

Fig. 7. Fluorescence images of stem cells cultured on KGM/chitosan matrix after 1 day. (A) KGM casting film, (B) KGM/chitosan casting film (the weight content of KGM was 60%), (C) chitosan casting film, (D) KGM electrospun membrane, and (E) KGM/chitosan fibrous membrane (the weight content of KGM was 60%).

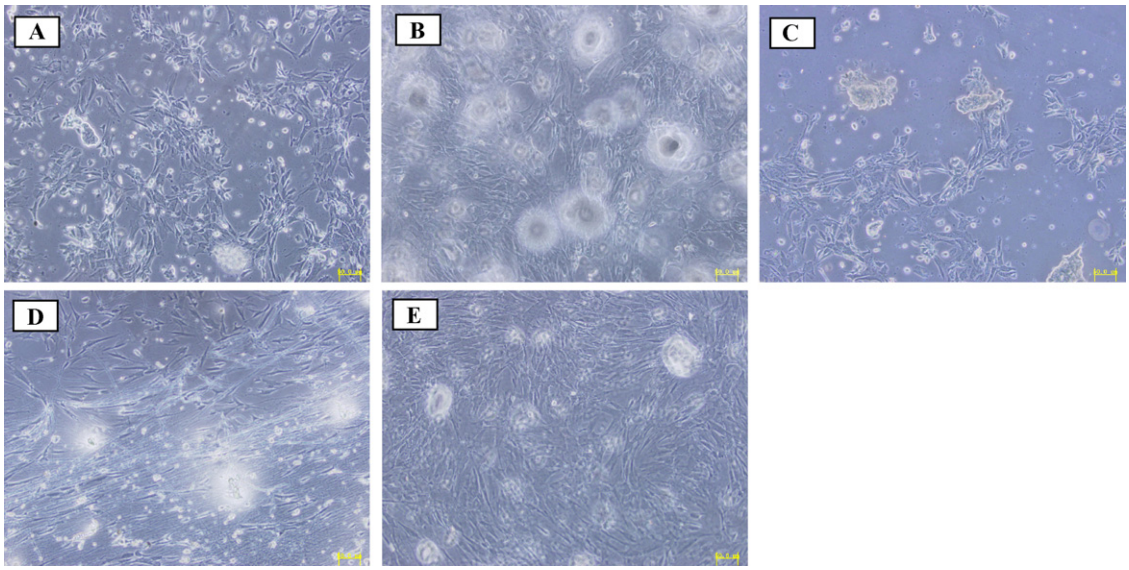

Fig. 8. Photographs of stem cells cultured on KGM/chitosan matrix after 1 day. (A) KGM casting film, (B) KGM/chitosan casting film (the weight content of KGM was 60%), (C) chitosan casting film, (D) KGM electrospun membrane, and (E) KGM/chitosan fibrous membrane (the weight content of KGM was 60%).

of these engineered scaffolds were in the same scale with those of the natural ECM. The high porosity of the electrospun nanofiber scaffolds provides enough space for the cell accommodation and an easy passage for the nutrient intake and metabolic waste exchange (Subbiah, Bhat, Tock, Parameswaran, & Ramkumar, 2005).

4. Conclusions

Konjac glucomannan (KGM), a water soluble natural polysaccharide with good biocompatibility and biodegradation has been electrospun from its aqueous solution. In addition, the bicomponent nanofibrous scaffolds of KGM and chitosan were prepared from their dilute acidic solution. The stability of KGM nanofibrous scaffolds was improved after NaOH aqueous solution treatment. The cell culture results show that bone mesenchymal stem cells adhere preferentially to the nanofibrous scaffolds of KGM and KGM/chitosan than the bulk films. Meanwhile, the addition of KGM contributes to improve the biocompatibility of chitosan material. It is expected that KGM and its composite nanofibrous scaffolds will have potential applications as a novel biomedical materials.

Acknowledgements

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