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Original Research

Genipin cross-linked electrospun chitosan-based nanofibrous mat as tissue engineering scaffold

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Abstract

Objective(s): To improve water stability of electrospun chitosan/ Polyethylene oxide (PEO) nanofibers, genipin, a biocompatible and nontoxic agent, was used to crosslink chitosan based nanofibers.

Materials and Methods: Different amounts of genipin were added to the chitosan/PEO solutions, chitosan/PEO weight ratio 90/10 in 80 % acetic acid, and the solutions were then electrospun to form nanofibers. The spun nanofibers were exposed to water vapor to complete crosslinking. The nanofibrous membranes were subjected to detailed analysis by scanning electron microscopy (SEM), Fourier transform infrared-attenuated total reflection (FTIR-ATR) spectroscopy, swelling test, MTT cytotoxicity, and cell attachment.

Results: SEM images of electrospun mats showed that genipin-crosslinked nanofibers retained their fibrous structure after immerging in PBS (pH=7.4) for 24 hours, while the uncrosslinked samples lost their fibrous structure, indicating the water stability of genipin-crosslinked nanofibers. The genipin-crosslinked mats also showed no significant change in swelling ratio in comparison with uncrosslinked ones. FTIR-ATR spectrum of uncrosslinked and genipin-crosslinked chitosan nanofibers revealed the reaction between genipin and amino groups of chitosan. Cytotoxicity of genipin-crosslinked nanofibers was examined by MTT assay on human fibroblast cells in the presence of nanofibers extraction media. The genipin-crosslinked nanofibers did not show any toxic effects on fibroblast cells at the lowest and moderate amount of genipin. The fibroblast cells also showed a good adhesion on genipin-crosslinked nanofibers.

Conclusion: This electrospun matrix would be used for biomedical applications such as wound dressing and scaffold for tissue engineering without the concern of toxicity.

Keywords: Chitosan, Cytotoxicity, Electrospinning, Genipin, Structural stability

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Introduction

Electrospun nanofibers have attracted many interests for different applications in recent years because of their unique properties such as high surface to volume ratio and high porosity (1). These nanofibers have been studied for various applications including membranes and filtration (2), sensors (3) and biomedical applications such as drug delivery (4), wound dressing (5), tissue engineering, (6) and biosensing (7). Among many synthetic and natural polymers electrospun to nanofibers, chitosan has attracted many interest especially for biomedical applications owing to its unique properties. Chitosan is a biopolymer composed of repeated β-D-glucosamine and N-acetyl-β-D-glucosamine monomers and is obtained from deacetylation of chitin, the second most abundant natural polymer in the world. Chitosan has good biocompatibility and biodegradability as well as various biofunctionalities including antithrombo-genic, hemostatic, and wound healing properties (8). Chitosan-based electrospun nanofibers have shown potential for many biomedical applications owing to their structural similarity to glycosaminoglycans, a component of extra cellular matrix (ECM), and morphological proximity to fibrous collagen structures in the ECM at the scale of nanometers (50–500 nm in diameter). The use of nanofibrous chitosan matrices is thus expected to mimic the natural ECM, in which cells can attach, proliferate, and differentiate (8, 9). Despite these desirable features, chitosan-based electrospun nanofibers are not stable in biological medium and they easily swell and lose their fibrous structure in contact with water. Therefore, chitosan-based nanofibers need to be crosslinked to maintain their structural integrity. Glutaraldehyde (GA) is a common reagent used for crosslinking electrospun chitosan nanofibers (10). However, GA is a toxic reagent and it is not suitable for application in biological systems (11). Hence, crosslinking of chitosan nanofibers with a low toxic reagent is desirable.Genipin is an aglycone of geniposide, which is derived from the fruits of Gardenia jasminoides Ellis. Because of its ability to react with free amino groups, genipin is used for crosslinking chitosan and proteins with free amino groups (12, 13). Meanwhile, Sung et al. showed that genipin might be about 5000–10000 times less cytotoxic than GA (14, 15). Chitosan in the forms of hydrogels (16) and microsphers (17) has been crosslinked by genipin. Genipin has also been used to crosslink electrospun gelatin (18), collagen (19) and silk fibroin/hydroxybutyl chitosan (20) nanofibers. In addition, pure electrospun chitosan nanofibers have also been crosslinked by genipin using trifluoroacetic acid (TFA), which is a well-known solvent for electrospinning of chitosan (21, 22). However, TFA is environmentally harmful, very toxic and corrosive, which makes its use very limited from an industrial point of view for biomedical applications (23). In the other hand, chitosan is not electrospinnable when other solvents such as acetic acid are used. A common approach to improve the electrospinnability of chitosan is blending it with easily electrospinnable polymer such as polyethylene oxide (PEO), polyvinyl alcohol (PVA), polylactic acid (PLA) and so on (9). Among all of these adding polymers, PEO is more preferable because it can lead to formation of chitosan nanofibers with the lowest amount of added polymer (23).To the best of our knowledge, genipin crosslinking of Chitosan/PEO electrospun nanofibers have not been reported yet. However, chitosan/PEO blend films have been crosslinked by genipin as potential membranes for potential medical applications (24). Explored in this study is the use of genipin for crosslinking electrospun chitosan/PEO nanofibers from aqueous acetic acid solution. Previously, two different approaches have been used

to crosslink pure chitosan nanofibers by genipin using TFA as solvent (21, 22). In one approach by Marjorie et al, genipin was added into chitosan/ TFA solution and mixed for 2 min immediately before electrospinning (21). In another approach by Frohbergh et al, chitosan nanofibers were first electrospun from a solution of chitosan dissolved in TFA and then the electrospun fibers were crosslinked with 0.1% (w/v) genipin dissolved in 1X PBS for 24 h (22).

In this study, a modification of Marjorie et al. approach was used to crosslink chitosan/PEO nanofibers with genipin. Crosslinking processes were done by adding various amounts of genipin into chitosan/PEO acetic acid solutions prior to electrospinning and then exposure of obtained nanofibers to water vapor at 30 °C for 24 h. The morphology, structure, water stability, swelling behavior, and cytotoxicity of genipin-crosslinked nanofibers were investigated as well and compared with those of cross-linked by GA.

The attachment of human fibroblast cells were also investigated on genipin-crosslinked nanofibers.

Materials and Methods

Materials

Chitosan (CS) (low molecular weight, degree of deacetylation 91.2 %) was purchased from Easter Groups (DongChen Co., Ltd, China). Polyethylene oxide (PEO) (MW 900 kD) was purchased from Acros Organics Co. Glacial acetic acid was purchased from Merck Chemical. Genipin (GP), methyl-2-hydroxy-9-(hydroxymethyl)-3-oxabicyclonona-4, 8-diene-5-carboxylate, was obtained from Challenge Bioproducts Co. Ltd. (Touliu, Taiwan). Glutaraldehyde was purchased from Panreac (Spain). RPMI medium and zolium bromide) from Sigma-Aldrich, USA. Human fibroblast (AGO-1522) were fetal bovine serum, FBS, were purchased from Gibco, USA and MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetra

Preparation of chitosan/PEO nanofibers

Chitosan solution (3.0 Wt. %) and PEO solution (3.0 Wt. %) were prepared separately by dissolving chitosan and PEO powders in aqueous acetic acid (80 V/V %) under magnetic stirring at 37 °C for 24 h.

The obtained solutions were then mixed together in weight ratio of CS/PEO, 90/10, as the required electrospinning solution with 3.0 Wt. % of total solid.

The electrospinning processes were carried out using Electroris (FNM, Tehran, Iran).

For every run, the polymer solution was placed into a 5 mL plastic syringe with a blunt-ended 18 G stainless steel needle. An aluminum foil was wrapped on the Electroris rotating drum as collector and was located at the distance of 12 cm from the needle.

A syringe pump fed the solution to the needle tip at the injection rate of 3.0 ml/h. The wire of a DC positive high voltage was connected to the metallic needle and the collector to the ground.

The applied voltage, drum speed, and electrospinning time were fixed at 20 kV, 200 rpm, and 1 h, respectively.

Crosslinking of chitosan nanofibers by genipin and glutaraldehyde

To obtain genipin-crosslinked chitosan nanofibers, 10 w/v % genipin solutions were prepared by dissolving genipin powder in pure ethanol.

Four different volumes of the genipin solution were added to a constant amount of the 3 Wt. % polymer solutions (CS/PEO, 90/10) at room temperature with constant stirring for 5 minutes.

The CS/genipin weight ratio in polymer-genipin solutions was 100/6, 100/3, 100/1 and 100/0.5 named CSg6, CSg3, CSg1 and CSg.5, respectively (Table.1).

Table 1 . Recipe used for preparing crosslinked and uncrosslinked chitosan nanofibers.

Experiment name	Total polymer content in solution (Wt. %)	CS/PEO weight ratio	Genipin content in solution (Wt. %)	CS/genipin weight ratio	Vapor used for crosslinking obtained electrospun mat
CSg6	2.95	9:1	$1.56*10^{-3}$	100:6	Water
CSg3	2.97	9:1	$0.8*10^{-3}$	100:3	Water
CSg1	2.992	9:1	$0.2*10^{-3}$	100:1	Water
CSg0.5	2.996	9:1	$0.13*10^{-3}$	100:0.5	Water
CSGA	3	9:1	0.0	100:0	25 % V/V glutaraldehyde in water
Uncrosslinked (CS)	3	9:1	0.0	100.0	No

These solutions were immediately electrospun under conditions described in previous section. The obtained genipin-contained mats were removed from the collector and immediately exposed to water vapor in a desiccator at 30 °C. To produce water vapor in the desiccator, a glass Petri dish was filled with 5 ml of distilled water and placed on the bottom of the desiccator. After 24 hours, the mats were washed with ethanol and then were dried at 37°C for 24 h, and maintained for further analysis. The CS/PEO electrospun mat was also crosslinked with GA as control using a common method (25).
The electrospun chitosan mat (without any added genipin) were exposed to vapor of 25 w/v % GA aqueous solution in desiccator at 30°C for 24 h. The resultant mat was then dried at 37°C for 24 h, and maintained for further analysis.The recipe used in this work for preparing chitosan crosslinked nanofibers is given in Table 1.

Characterization of chitosan nanofibers

The size and morphology of produced nanofibrous mats were analyzed using scanning electron microscopy (SEM) (ZEISS DSM 960A Oberkochen, Germany). A small section of each nanofibrous mat was sputtered with a thin layer of gold and then analyzed by SEM.
The Fourier transform infrared attenuated total reflection spectroscopy (FTIR-ATR) spectra were obtained using FTIR instrument (Equinox 55, Bruker, Germany) in the range of 2500–500 cm^{-1}.
The water stability of nanofibers was investigated by observing SEM images of electrospun nanofibers before and after immerging in PBS (pH=7.4) at room temperature for 24 h.
The swelling degree of electrospun mats was determined by a gravimetric method. The electrospun mats were immerged in PBS (pH=7.4) at room temperature for 24 h. The samples were then taken out, the excess surface water was removed by filter paper, and the swelled mats were weighed. The swelling ratio was measured as following equation:

$$\text{swelling ratio } (\%)=\frac{W_s-W_0}{W_0}\times 100$$

Where W_s denotes the weight of the swelled mat and W_0 denotes the weight of the mat in its dry state after 24 immerging in water.

MTT assay and cell viability

Cytotoxicity of the crosslinked mats was examined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay of cell viability according to ISO 10993-5 standard test method. The electrospun mats were cut into 1 × 1 cm square and sterilized by UV irradiation for 2 h. Samples were put into 15-ml falcons

and 2 ml of culture medium (RPMI) was added into each sample. The samples were then incubated at 37 °C for 3 incubation time intervals, i.e., 1, 3 and 6 days, and the extracts of each sample were collected for cell culture. Fresh culture medium was used as negative control. Human fibroblasts (AGO-1522) were seeded in 96-well tissue culture plate at a density of 1.2×10^4 cells/well in RPMI medium supplemented with 10% fetal bovine serum, and incubated at 37 °C under 5% CO_2/95% air condition for 24 h. After that, the culture medium was removed and replaced with the as-prepared extraction medium, supplemented with 10% fetal bovine serum and incubated for another 24 h, then the extraction medium was removed and 100 µL MTT solution (0.5 miligram/1 mililiter) was added to each well. After incubation at 37 °C and 5% CO_2 for 4 h, the MTT solution was removed and 100 µL isopropanol was added to dissolve formazan crystals, formed by mitochondrial succinate dehydrogenase of alive cells from MTT. After 20 min incubation at 37 °C, the optical density of the formazan solution was detected by an ELISA reader (AWARENESS TECHNOLOGY, Stat fax-2100, USA) at 570 nm of wavelength. The mean absorption of each sample (10 well per each experiment) was divided to mean absorption of the control to calculate the percent of cell viability for each sample. The test was repeated 5 times for each sample.

Cell seeding and adhesion

The genipin-crosslinked mat (CSg1) were cut into 1 × 1 cm square and sterilized by UV irradiation for 2 h. The samples were then washed three times with sterile PBS prior to transfer to individual 12-well tissue culture plates. The number of 15000 human fibroblasts (AGO-1522), suspended in RPMI, were seeded on the samples, cultured for 3 h and then 1 ml of RPMI supplemented with 10% FBS were added to each well. After 24 h of culture, cellular constructs were harvested, rinsed twice with PBS to remove nonadherent cells, and subsequently fixed with 2.5 % glutaraldehyde at 4 °C for 4 h. After that, the samples were dehydrated through a series of graded ethanol solutions and air-dried overnight. Dry samples were sputtered with gold for observation of cell morphology on the surface of the scaffolds by SEM.

Statistical analysis

Analysis of variance (ANOVA) was used to compare the swelling ratio of chitosan nanofibers. The number of samples for each group was 5 and the data were represented as mean ± SD. The significance was considered as P.value < 0.05. The data of MTT assay were analyzed using one sample t test with bonferroni correction and differences from control were considered statistically significant at P.value < 0.01.

Results and Discussion

Appearance and morphology of mats

In this work, genipin was used as a safe and biocompatible crosslinking agent instead of toxic but common crosslinking agent of chitosan nanofibers (i.e., glutaraldehyde).

The polymer solutions with different content of genipin (i.e., CSg.5, CSg1, CSg3, and CSg6) were electrospun and subsequently, the mats were immediately exposed to water vapor at 30°C for 24 h. Gross changes in the color of the samples after being exposed to water vapor were observed. The digital photographs of these mats compared to uncrosslinked (CS) and GA-crosslinked (CSGA) mats are shown in Figure 1.

The color of genipin-crosslinked chitosan mats changed from white to bluish green and dark blue depending on the genipin content (Figure 1. CSg.5, CSg1, CSg3 and CSg6), While, the color of GA-crosslinked mats (CSGA) turned from white to brown yellowish (Figure 1. CSGA).

The mats with higher amount of genipin

(CSg6, CSg3) represented dark bluish color in comparison with mats with lower genipin content (CSg1, CSg.5) displaying bluish green color.

These color changes are associated with chitosan derivatives produced by the reaction of genipin with amino groups of chitosan (12). No color change were observed for the genipin contained nanofibers which not exposed to water vapor (data are not shown).

The morphology of the electrospun mats was investigated through the SEM micrographs.

The uncrosslinked chitosan mat had continuous and uniform nanofibrous structure (Figure 2a).

After crosslinking by various amount of genipin the total fibrous structure of the chitosan mats were kept constant, but there were a few structural deformations as shown in Fig. 3a, b, c, d, where a, b, c, and d are corresponded to Csg.5, CSg1, CSg3, and CSg6, respectively.

In comparison with uncrosslinked mats, the genipin-crosslinked mats showed fusion of adjacent nanofibers. On the other hand, crosslinking by glutaraldehyde caused no remarkable change in morphology of chitosan nanofibers (Figure 3e).

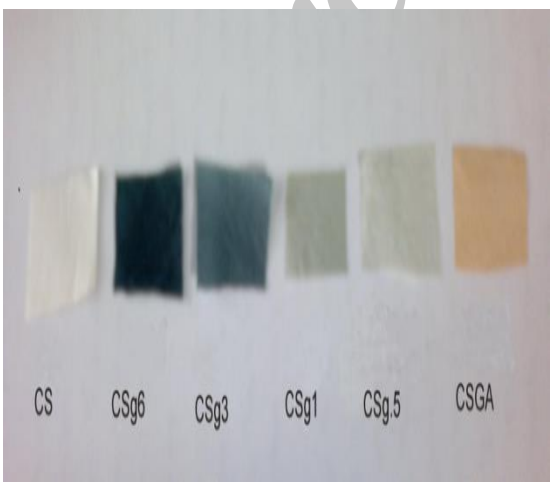

Figure1. digital photographs of uncrosslinked (CS), GA-crossliked (CSGA) and genipin-crossliked (CSg6,CSg3, CSg1, CSg.5) electrospun chitosan mats.

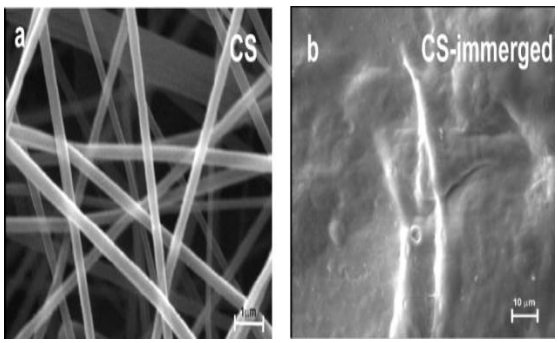

Figure 2. SEM images of uncrossliked nanofibers, (a) before and (b) after immerging in PBS (pH=7.4).

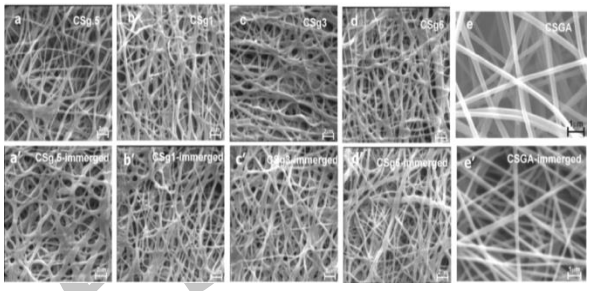

Figure 3. SEM images of genipin-corsslinked and GA-crosslinked chitosan nanofibers before (a, b, c, d, e) and after (a', b', c', d', e') immerging in PBS (pH=7.4).

Water stability and swelling behavior of electrospun mats

Uncrosslinked electrospun chitosan mats lose their fibrous structure in contact with water owing to their high swelling ratio and solubility in aqueous media (26). As shown in Figure 2b, the uncrosslinked chitosan mat completely lost its fibrous structure after immerging in water. This behavior of chitosan nanofibers makes them inappropriate for applications in aqueous media such as biological systems. Crosslinking is a way used to improve water stability of chitosan nanofibers (26). To solve this problem we used a non-toxic agent, genipin, to crosslink chitosan mats. The SEM images of crosslinked chitosan nanofibers after 24 h immerging in PBS (pH=7.4) are shown in Figure 3. The SEM images indicate that crosslinking of nanofibers with genipin (Figure 3a', b',c',d') or GA (Figure 3e') preserved the fibrous structure and morphology of nanofibers immerged in water. Crosslinking by genipin makes chitosan

structure stable in aqueous media by binding amino groups of chitosan (12). It is necessary to mention that exposure to water vapor is essential for genipin contained nanofibers to retain their fibrous structure after immerging in water. In other words, the genipin contained nanofibers which were not exposed to water vapor lost their fibrous structure after immerging in water (data are not shown). To investigate the water absorption capability of crosslinked nanofibers, the swelling behavior of nanofibers was studied. The more swelling ratio (%) related to the more water absorption capability.

The swelling ratio (%) of chitosan electrospun mats is stated in Figure 4.

It can be seen that the swelling ratio of chitosan mats did not decrease significantly using genipin as crosslinking agent, while crosslinking by GA reduced the swelling ratio significantly in comparison with uncrosslinked mats (P.value < 0.05).

So, unlike crosslinking by GA, crosslinking by genipin does not decrease the water absorption capability of chitosan nanofibers.

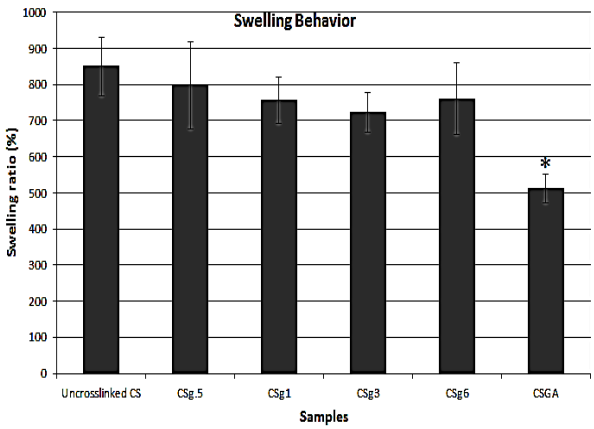

Figure 4. Swelling ratio of uncrosslinked and crosslinked chitosan nanofibers after 24 immerging in water. * represents significant difference from uncrosslinked CS (P.value < 0.05). Data are shown as mean ± SD (n=5).

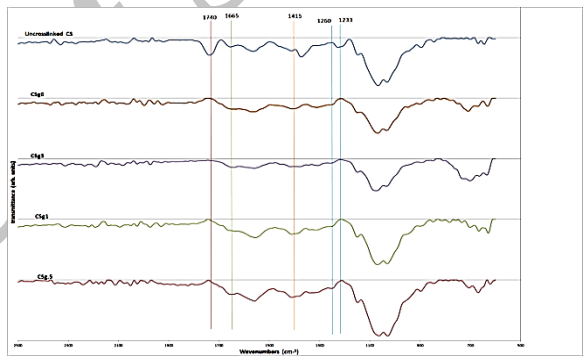

Figure 5. FTIR-ATR spectrum of uncrosslinked and genipin-crosslinked (CSg.5, CSg1, CSg3, CSg6) chitosan nanofibers.

FTIR-ATR spectroscopy

When CS systems are crosslinked with genipin, conformational changes occur as a result of structural rearrangement of chains to form covalent bonds (27).

To investigate the chemical changes of chitosan after crosslinking by genipin,

The FTIR-ATR analysis of uncrosslinked and genipin-crosslinked chitosan nanofibers was carried out as shown in Figure 5.

Comparing with FTIR spectrum of uncrosslinked chitosan nanofibers, FTIR spectrum of genipin-crosslinked chitosan nanofibers revealed the crosslinking of chitosan by genipin (Figure5).

The C=O stretching band of chitosan amide I at 1665 cm−1 (28) became broad after crosslinking due to overlapping with C=C stretching in cyclic structure of genipin at 1628 cm− 1 (20).

The amino groups of chitosan react with genipin and form amide linkage and heterocyclic amine in genipin-crosslinked chitosan network (12). The C=O stretching absorption band of new formed amide at 1650 (12) again overlaps with amid I absorption band of chitosan and makes it become broader. A new broad peak that was appeared around 1415 cm^{-1} after crosslinking by genipin, indicated the presence of ring-stretching of heterocyclic amine (12).The C–N stretching of amide III at 1233 cm^{-1} (27) shifted to 1260 cm^{-1} after crosslinking by genipin.

The peak at 1740 cm^{-1} corresponded to

the presence of carboxylic groups in electrospun chitosan fibers (29) disappeared in the genipin-crosslinked chitosan fibers. This can be due to the exit of carboxylic groups from genipin-added nanofibers when they expose to water vapor.

Cytotoxicity of crossliked nanofibers

The cell viability test using MTT was used to evaluate cytotoxicity of the crosslinked chitosan mats. Figure 6 represents the percent of cell viability after culturing the cells with extraction media of mats for three different incubation time intervals for each sample (1, 3, and 6 days). The values were represented as mean ± SD (n=5). For the chitosan mats crosslinked by glutaraldehyde (CSGA), the cell viability was significantly lower than that of the control for all incubation time intervals (*P*.value < 0.01). The cell viability of this sample was around 80 %. For CSg.5, CSg1, and CSg3 the cell viability (%) was similar to those of the control (fresh culture medium), around 100 %.

However, The CSg6 showed significantly lower cell viability (around 80 %) than the control (*P*.value < 0.01). This difference was seen for all incubation times.

The lower cell viability of CSg6 can be attributed to the excess amount of residual genipin that may not react with chitosan and released to the medium after incubation of samples in the medium. From Figure 6, it could be also observed that for all crosslinked samples, the cell viability was not decreased by increasing incubation time of samples.

This result implies that toxic agents were released at first 24 h incubation time, and after that no more toxic agent were released by increasing incubation time (from 1 day to 3 and 6 days). It could be resulted from MTT assay that crosslinking chitosan nanofibers with lower amount of genipin (3 Wt. % or lower by weight of chitosan) cause no cytotoxicity.

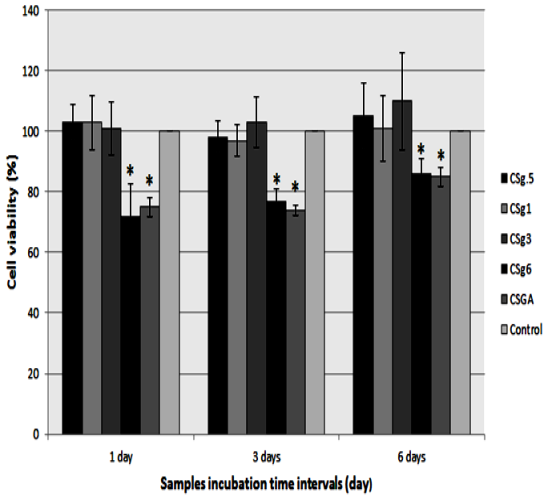

Figure 6. Cell viability of fibroblasts in presence of extraction media of crosslinked chitosan nanofibers in three incubation time intervals (1, 3, and 6 days). * indicates significant difference from the control (P.value < 0.01). Data are shown as mean ± SD (n=5).

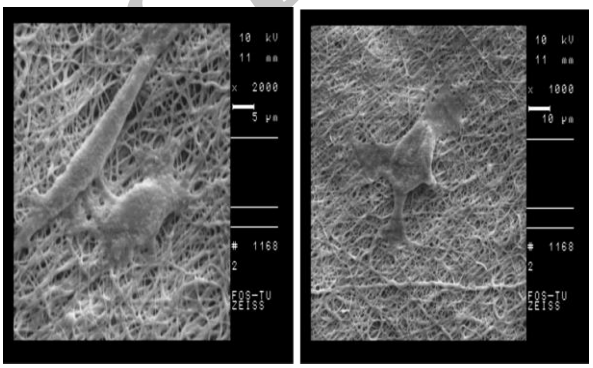

Figure 7. SEM images of fibroblasts (AGO-1522) seeded on nanofibrous membrane of genipin-crosslinked (CSg1) after 24 h culture.

Cell adhesion

The genipin-crosslinked nanofibrous membrane (CSg1) was used for cell adhesion study. Figure 7 shows SEM images of fibroblasts grown on the cross-linked nanofibers after 24 h cell culture. Cells were attached to the surfaces by discrete filopodia and exhibited a normal morphology on the surface, which was due to the large surface area available for cell attachment.

Conclusion

The chitosan/PEO nanofibers were successfully crosslinked by a biocompatible and nontoxic crosslinking

agent, genipin, which leads to structural stability of chitosan nanofibers in aqueous media. In addition, the water absorption capability of chitosan nanofibers was not decreased after crosslinking by genipin. Genipin-crosslinked nanofibers also showed no cytotoxicity unless at high amount of genipin. Because of no cytotoxicity and bio compatibility of genipin-crosslinked chitosan nanofibers, these nanofibers are suggested for biomedical application such as tissue engineering and wound dressing instead of glutaraldehyde-crosslinked nanofibers.

Acknowledgements

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