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Original Article

Preparation and *in vitro* characterization of electrospun scaffolds composed of chitosan, gelatin and 58S bioactive glass nanoparticles for skin tissue engineering

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Abstract

Background and aims: The presence of an appropriate scaffold at the wound site could significantly improve the healing process. In this study, we aimed to prepare a biomimetic nanocomposite scaffold composed of chitosan, gelatin, and 58S bioglass nanoparticles for skin tissue engineering.

Methods: The nanocomposite scaffolds composed of chitosan, gelatin, and 58S bioglass nanoparticles were fabricated through electrospinning process. Then the cell viability assay was performed in order to evaluate the biological properties of the membranes. The optimum concentration of bioglass nanoparticles was determined for further studies. *In vitro* characterization was also performed to evaluate physicochemical properties of the scaffolds.

Results: The chitosan/gelatin scaffold containing 2% of 58S bioglass nanoparticles showed no cell toxicity, and the dermal fibroblasts were found capable of proliferation on the membrane. The *in vitro* results obtained from the scanning electron microscopy (SEM), attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), and porosity tests demonstrated the appropriate properties of the membrane as a scaffold for skin regeneration.

Conclusions: It was concluded that a chitosan-gelatin membrane containing 2% of 58S bioglass nanoparticles had the potential to function as a scaffold to accelerate wound healing due to its suitable properties, such as high porosity, high surface/volume ratio, and excellent biocompatibility.

Keywords: Chitosan, Gelatin, Bioactive glass, Wound healing, Tissue engineering

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Introduction

Skin is the largest organ of the body and the first line of defense against external pathogens. Large skin lesions may lead to blood/water losses, bacterial invasion, and infections (1). The current clinical therapeutic methods include skin grafting techniques. Although autologous grafting as the main treatment provides a good chance for healing, it cannot be always used due to its many limitations such as patients suffering, scar formation, slow rate of healing, and limited donor sites (2). In this regard, allografts and xenografts are considered as alternative methods of treatment. Moreover, immune rejection and disease transmission (e.g., zoonosis and viral infections) are the main limitations of these methods (3). The limitations and insufficient potentials of the current treatments have encouraged the researchers to investigate alternative methods.

Tissue engineering as a regenerative therapeutic approach uses the principles of engineering and biology to maintain the structure as well as to improve the function of damaged tissues. Notably, an appropriate substrate for cell attachment and migration could mimic extracellular matrix and enhance the tissue regeneration (4). There are different methods that could be applied to produce suitable

scaffolds for skin tissue engineering. Electrospinning has been recently introduced as a new method to produce such constructs. Accordingly, this technique provides fibrous scaffolds that could increase cell attachment, migration, proliferation, and differentiation due to its high ratio of surface/volume and porosity (5, 6).

Chitosan (CS) and gelatin (GEL) are natural-based biopolymers that are widely used for wound healing due to their biocompatibility, cell-adhesive properties, and low immunogenicity (7). Electrospun CS/GEL-based nanofibrous scaffolds have been used for various biomedical applications in the last decade (8). Furthermore, bioactive nanomaterials could be easily incorporated into these scaffolds to improve their biocompatibility (9).

Bioactive glasses (BGs) are those designed silica-based glasses in which the three-dimensional SiO_2 network is modified by the addition of certain ratios of CaO, Na_2O, and P_2O_5. Different BGs are produced by mixing the different ratios of these oxides. Furthermore, additional oxides may also be added to this mixture in order to improve the specific biological and/or physicochemical properties (10). Bioglass nanoparticles (BG-NPs) have offered more advantages such as faster dissolution of ions, higher specific surface area, and better biocompatibility compared to conventional BGs. Moreover, it has been shown that they have the capability of inducing angiogenesis during the wound healing process (11,12).

Wound healing – chronic wound healing, in particular, is currently considered as a critical issue. Chronic wounds often plague patients and medical systems, and cause huge losses to the social economy. Pre-clinical studies have demonstrated the potentials of tissue engineering methods in wound healing applications. In this study, nanofibrous scaffolds composed of CS, GEL, and 58SBG-NPs were produced. *In vitro* characterization of the scaffolds was performed to find out if they were potent scaffolds to be used for wound healing.

Materials and Methods

Preparation of 58SBG-NPs

The 58SBG-NPs were synthesized using the sol-gel method (13). Hydrochloric acid (HCl) was diluted with deionized water at a mole ratio of 8:1. Thereafter, tetraethyl orthosilicate (TEOS) (Sigma Aldrich, USA, CAS No. 78-10-4) was added to the diluted HCl and mixed for 30 minutes. Triethyl phosphate (TEP) (Sigma Aldrich, USA, CAS No. 78-40-0) was added to this mixture and was mixed for another 30 minutes. Subsequently, calcium nitrate (Sigma Aldrich, CAS No. 13477-34-4) was added to the stirring acid solution. After an hour, ammonia (1.0 molar) was added to the solution drop-wise and then was stirred at 1200 rpm. The gel phase was immediately formed. The obtained gel was heated for 180 minutes at 750°C. Finally, the synthetized 58SBG-NPs (58%SiO2-33% CaO-9% P_2O_5) were analyzed by dynamic light scattering (DLS) and scanning electron microscopy (SEM).

Characterization of 58sBG-NPs

SEM was used to analyze the size and morphology of the synthetized nanoparticles. Two mg of 58SBG-NPs powder was dispersed by sonication in 20 mL of deionized water. A single drop of the suspension was placed on a piece of aluminum foil and then dried for an hour at 37°C. The sample was coated with gold using a sputter coater (Quorum Q150R ES, Quorum Technologies Ltd, UK) and then SEM image was obtained by using a S3400 Scanning Electron Microscope (Hitachi, Japan) and applying an accelerated voltage of 20 kV.

Average size and size distribution of the 58SBG-NPs were determined using DLS technique (Horiba Scientific SZ-100 Nanoparticle Analyzer, Kyoto, Japan)

Preparation of the electrospun scaffolds

The CS and GEL solutions were prepared and electrospun in a dual pump way. By dissolving CS in 80% acetic acid (20% H_2O), 3% w/v of CS solution was obtained. Notably, GEL solution consisted of 18% w/v of Gel polymer in 80% acetic acid. The CS and GEL solutions were then placed in crystal syringes separately. CS was electrospun at 140 mm distance, 25 kV voltage, and 1 mL/h flow rate. GEL was simultaneously electrospun at 120 mm distance, 28 kV voltage, and 1 mL/h flow rate. Different concentrations (i.e., 0%, 0.5%, 1%, 2%, 3%, and 4% w/w) of 58SBG-NPs were added to the CS and GEL solutions to prepare incorporated scaffolds. Cell viability tests were performed to determine the optimal concentration of the nanoparticles. Then *in vitro* characterization of the membrane was performed to evaluate its potential as an engineered scaffold for skin tissue engineering applications.

Crosslinking

The scaffolds were dried for 48 hours at room temperature and then cross-linked by exposure to glutaraldehyde vapor for 12 hours. Thereafter, the cross-linked membranes were immersed in glycine for 1 hours in order to deactivate the remaining glutaraldehyde.

Cell viability

Rat dermal fibroblasts (passage 4) were used to evaluate cell compatibility of the scaffolds incorporated with different concentrations of 58SBG-NPs. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Mfr. No. Gibco™ 31600083) that was enriched with 10% fetal bovine serum (FBS) (Gibco, Mfr. No. Gibco™10082139) and 1% penicillin/streptomycin (Gibco, Mfr. No. Gibco™ 15140122). The cells were cultured at 37°C with 5% CO_2 and 94% humidity. To obtain the best concentration of 58SBG-NPs, CS-GEL scaffolds containing 0%, 0.5%, 1%, 2%, 3%, and 4 w/w% of nanoparticles were fabricated. Scaffolds (n = 3 for each concentration) were placed in 96-well plates and 5×10^3 cells were then seeded on each membrane. The control wells contained the same cell density and amount of culture media as other wells

did. There were no scaffolds in the control wells. The CCK-8 assay was performed on the 1st, 3rd, and 7th days of incubation, and 10 µL of CCK-8 solution was added to each well. The cells were incubated for 4 hours and then the absorbance of each well was read using plate reader at 450 nm (14). Finally, the scaffold containing the optimal concentration of nanoparticles was selected for further analyses.

Scanning electron microscopy

SEM was used to analyze the quality and morphology of the electrospun fibers as well as the suitability of the crosslinking procedure. The crosslinked scaffolds were then coated with gold using a sputter coater and, afterward, SEM image was obtained by using a AIS2300C Scanning Electron Microscope.

ATR-FTIR

To confirm the composition of the electrospun scaffolds, attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) analysis was conducted. The spectra were obtained using a Thermo Scientific iS10 FTIR spectrophotometer, ranged from 4000 cm^{-1} to 400 cm^{-1}; the spectral resolution was 4 cm^{-1}.

Porosity

Liquid displacement method was used to evaluate the membranes porosity (15). Scaffolds were weighed before and after the immersion process (1 hour in EtOH). The porosity of scaffolds was calculated applying the following formula:

$$\text{Porosity}\ (\%) = (W_s - W_d / D_{EtOH} \times V_{scaffold}) \times 100$$

(W_s: Swollen scaffold weight, W_d: Dry scaffold weight, D_{EtOH}: Density of the ethanol, and $V_{scaffold}$: Volume of the swollen scaffold). Three replicates were used for each scaffold and the averages were reported.

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's HSD post hoc test were performed to evaluate the quantified outcomes for determining statistical differences (SPSS 16.0 software). The $P < 0.05$ was considered as statistically significant.

Results

SEM and DLS analyses of the synthesized 58SBG-NPs

The results obtained from SEM and DLS revealed the morphology, average size, and size distribution of the 58SBG-NPs. The average size of the nanoparticles was 67 nm with a size distribution of about 58-95 nm. The SEM image also confirmed the nanosize of the particles (Figure 1).

SEM analysis of the electrospun scaffolds

The SEM images showed the randomly oriented electrospun nanofibers of the scaffolds. The images also confirmed the suitability of the crosslinking procedure (Figure 2).

Cell viability

The viability of rat dermal fibroblasts was assessed over the 1st, 3rd, and 7th days from cell seeding process in the presence of scaffolds using CCK-8 assay (Figure 3). Scaffolds containing 0.5%, 1%, 2%, and 3% of 58SBG-NPs showed no significant cytotoxicity ($P > 0.05$). Cell viability decreased significantly after 7 days for the 4% group compared to the control group ($P = 0.001$). It is noteworthy that those scaffolds containing 2% of 58SBG-NPs showed the best compatibility compared to the other groups. Therefore, CS-GEL scaffold containing 2% of 58SBG-NPs was selected for performing further physico-chemical studies.

ATR-FTIR

The ATR-FTIR spectra of CS-GEL and CS-GEL/58SBG-NPs scaffolds are presented in Figure 4.

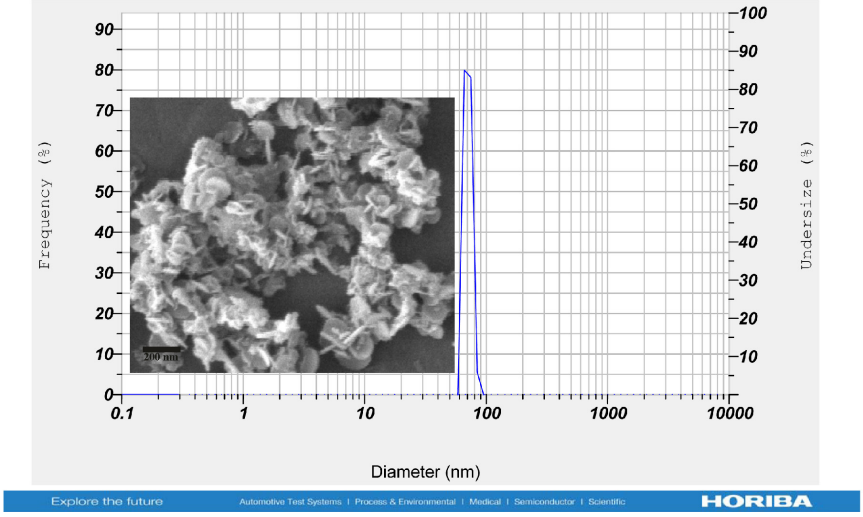

Figure 1. SEM image and particle size distribution of 58SBG-NPs.

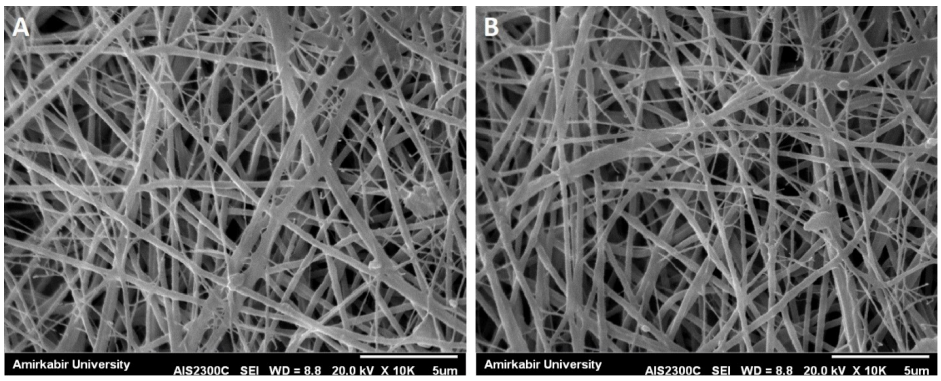

Figure 2. SEM of the CS-GEL (A) and CS-GEL/58SBG-NPs (B) crosslinked fibers.

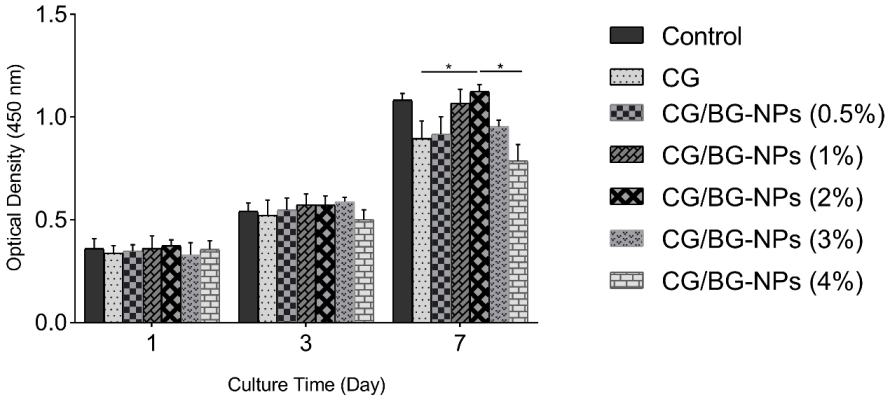

Figure 3. CCK-8 Assay for rat Dermal Fibroblasts. Data are shown as mean ± SD (n = 3), *P < 0.05.

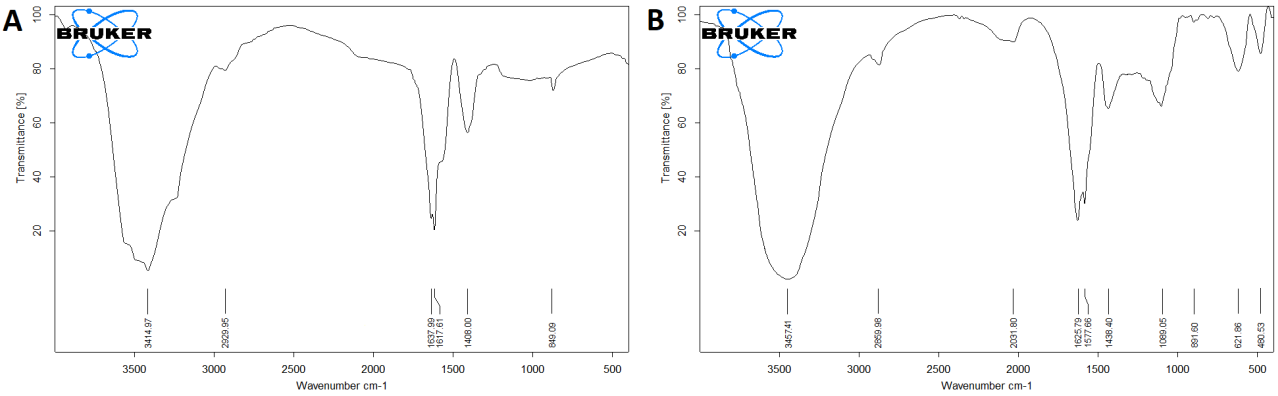

Figure 4. ATR-FTIR Spectra of CS-GEL (A) and CS-GEL/58SBG-NPs (B) scaffolds.

Correspondingly, the spectrum of CS-GEL/58SBG-NPs showed the characteristic bands of CS and GEL as well as the incorporated BG-NPs bands. Table 1 shows the characteristic bands of CS-GEL and CS-GEL/58SBG-NPs scaffolds.

Porosity

Porosities of the CS-GEL and CS-GEL/58SBG-NPs scaffolds were determined using the ethanol displacement method (15). In addition, the CS-GEL membranes revealed a porosity of 71.69 ± 5.42 %. Incorporation of 58SBG-NPs slightly increased the porosity of the membrane up to 75.51 ± 4.86 %; however, this increase was not statistically significant (P = 0.207).

Discussion

In this study, electrospinning was used to fabricate highly porous scaffolds for skin tissue engineering. The porous structure of the electrospun scaffolds could facilitate the exchange of gas and nutrients as well as support cell proliferation and migration (15). Biocompatibility is the most important feature determining the suitability of a polymer for biomedical applications (16). In the present study, scaffolds were fabricated using highly biocompatible materials, CS, and GEL as the main supportive biomaterials as well as BG-NPs as the bioactive agent.

The 58SBG-NPs were synthesized using the sol-gel method. The SEM image and the result of the DLS analysis showed the nanosize of the 58SBG-NPs, which made them suitable for incorporation into electrospun

Table 1. Characteristic bands of CS-GEL and CS-GEL/58SBG-NPs scaffolds

CS-GEL	CS-GEL/58SBG-NPs
3414.97 (O—H)	3457.41 (O—H)
2929.95 (Amide B)	2859.98 (Amide B)
1637.99 (Amide I)	1625.79 (Amide I)
1617.61 (Amide II)	1577.66 (Amide II)
1408.00 (Absorbed molecular water)	1438.40 (Absorbed molecular water)
	1089.05 (Si—O—Si)
	891.60 (CO_3^{-2})
	621.86 (PO_4)
	480.53 (PO_4)

fibers (Figure 1). Subsequently, ATR-FTIR was performed to investigate the incorporation of 58SBG-NPs into the electrospun fibers. Also, the presence of bands at 1089.05 cm^{-1} (Si—O—Si), 891.60 cm^{-1} (CO_3^{-2}), 621.86 cm^{-1} (PO_4), and 480.53 cm^{-1} (PO_4) demonstrated the incorporation of 58SBG-NPs into the CS-GEL fibers (Figure 4). Incorporation of the nanoparticles into the CS-GEL fibers decreased the diameter of the fibers; however, it was not significant. Thinner fibers were fabricated due to a slight decrease in the viscosity of the solution. Other previously performed studies had reported a similar effect on the diameter of electrospun scaffolds when adding nanoparticles or other agents to the solution (15,17,18). Furthermore, the nanofibers produced in the current study had an average diameter comparable to that of the collagen fibers present in native ECM (60–400 nm) (Figure 2). The CS-GEL and CS-GEL/58SBG-NPs scaffolds were cross-linked by exposure to glutaraldehyde vapor. SEM images of the cross-linked scaffolds confirmed the suitable method of crosslinking (Figure 2).

Biological assessments and *in vitro* characterization of membranes were performed to evaluate their suitability for wound healing applications. According to the CCK-8 viability assay, the optimum concentration of 58S5BG-NPs was 2% w/w for rat dermal fibroblast cells (Figure 3). Interaction between cells and ECM components is critical for stabilizing the three-dimensional structure and retrieving tissue function during tissue remodeling in wound healing process (19,20). An appropriate dermal scaffold should induce cell adhesion, proliferation, and migration to promote the wound healing process. Fibroblast cells are responsible for the synthesis of collagen and ECM bio-macromolecules as well as for the secretion of adherent proteins like fibronectin (21,22). Therefore, the cell compatibility of the scaffolds keeps the normal rate of ECM synthesis, which is necessary for wound healing process. It can be stated that the enhanced proliferation of the rat dermal fibroblasts on the CS-GEL membrane containing 2% w/w of 58SBG-NPs demonstrates the suitability of the membrane for wound healing applications.

The porosity of scaffolds is important for accommodating cells as well as facilitating their migration and the exchange of gas and nutrients (23). Incorporating 58SBG-NPs slightly increased the porosity of the membranes, which could have been attributed to lower diameter of CS-GEL/58SBG-NPs fibers and wider spaces between them compared to CS-GEL membranes. Generally, overall porosity values between 60% and 90% are appropriate for cell migration, ECM production, and gas and nutrient exchange to improve wound healing process (24,25). In the present study, membranes displayed porosity values within the mentioned range.

Conclusions

According to the CCK-8 assay results, the CS-GEL scaffold containing 2% of 58SBG-NPs was the most biocompatible one for dermal fibroblasts. The assessment of the physicochemical properties of the scaffold also demonstrated its potential for healing wounds. The ATR-FTIR spectra showed the characteristic bands of CS, GEL, and BG-NPs, proving the successful incorporation of 58SBG-NPs into the nanofibrous scaffold. SEM images revealed the randomly oriented fibers of CS-GEL and CS-GEL/58SBG-NPs; the images also confirmed the suitability of the crosslinking procedure. The high porosity of the scaffold facilitated the exchange of nutrients and gas as well as cell proliferation and migration; it also provided adequate space for angiogenesis and new ECM formation.

Overall, the CS-GEL electrospun scaffold containing 2% of BG-NPs showed appropriate physicochemical and biological properties to be used as a scaffold for healing wound. It was recommended that further studies be conducted to investigate the incorporation of ECM components and growth factors to find out if they could induce a synergistic effect on improving wound healing process.

Authors' Contributions

Conception and design, acquisition of data, analysis and interpretation of data were performed by HN, MB and MK. Drafting of the manuscript was done by HN. Critical revision of the manuscript for important intellectual content and statistical analysis were carried out by MB, MK and KM. The final draft was approved by ES, SA and MS. All authors read and approved the manuscript.

Conflict of Interests

Authors declare no conflict of interests.

Ethical Approval

The study protocol was approved by the Research Ethics Committee of Shahrekord University of Medical Sciences (IR.SKUMS.REC.1398.161).

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