Supporting Information

Fabrication of poly(vinyl alcohol)/gelatin biomimetic electrospun nanofibrous composites and bioactivity assessment of it for bone tissue engineering

1. Materials

Polyvinyl alcohol (PVA) with an average molecular weight of 77,500 g/mol supplied by Loba Chemie. Gelatin (type B, derived from bovine skin) and glutaraldehyde (GA) (20% aqueous solution) purchased from Merck. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (antibiotics), and trypsin-EDTA purchased from Biowest. The osteogenic differentiation medium and Alizarin red provided by Bio-IDEL. Other chemical reagents purchased from Merck and used as received without further purification.

2. Instruments

The specific chemical groups of the pure PVA, pure gelatin, and crosslinked PVA/Ge nanocomposite were analyzed by FTIR (Perkin Elmer FTIR Spectrum X1, USA) with a KBr powder. The scaffold samples were analyzed at the wavelength range of 400-4000 cm^{-1}. The morphology of the nanofibrous and cell seeded scaffolds was examined using SEM (LEO 1430VP) that was operated at the acceleration voltage of 15 kV. Before analysis, samples were sputter-coated with gold. The thermal stability of the nanocomposite scaffolds was investigated by TGA (Linseis STA PT-1000) analyses. The TGA curves were obtained under a nitrogen atmosphere at a flow rate of 10 °C/min. Approximately 15 mg of the sample was heated from 30 to 600 °C. The nanofibers fabricated using an electrospinning set (Nano Fanavaran Meghyas Co., Iran).

3. Fabrication of crosslinked PVA/GE electrospun scaffold

PVA solutions (12 w/v %) were dissolved in deionized water at 80 °C with constant stirring for at least 3 h. 12 w/v % GE solutions were prepared in a solvent consisting of deionized water: acetic acid 1:9 at ultrasonic bath under gentle stirring for 30 min. Then, the GE solution was added to the PVA solution at specific volumes to obtain a PVA/GE volume ratio of 50:50. The mixture was then stirred for 15 min at an ultrasonic bath before electrospinning to obtain a homogenous solution (1). The PVA/GE nanofibrous scaffolds were fabricated using an electrospinning method. Briefly, for electrospinning, the homogenous hybrid solutions were placed into a 20 mL syringe fitted to a needle. A syringe pump was set to a constant feeding rate of 0.5 mL/h. The voltage power supply was controlled at 22 kV while the collecting rate was 200 rpm. A piece of aluminum foil was utilized as a collector and placed 10 cm from the syringe tip. The electrospinning procedure was performed at room temperature (2). For enhancement of resistance in water solutions, nanofibrous scaffolds were crosslinked by GA vapor in two steps. In the first step of crosslinking, PVA/GE fibrous scaffolds were hung on the edge of the beaker. GA (20%) was added into the beaker and covered with aluminum foil for 24 h to crosslink the polymeric chains through the evaporation of GA at 30 °C. Then, the GA treated PVA/GE mat was dried at 120 °C for 2 h in an electric oven to remove residual GA and enhance the crosslinking density. In the second step, GA treated scaffolds were placed for 12 h in a 20 mL solution of methanol/GA (20%). Then, the crosslinked PVA/GE scaffolds were dried at 120 ˚C for 12 h in an electric oven to remove extra methanol and non-reacted GA (2).

4. Contact angle test

The surface hydrophilicity or hydrophobicity of scaffolds was examined by measuring its water contact angle. To do this, a drop of deionized water in a volume of 4 µL was carefully placed on the surface of dry samples. The water droplet was manually dropped onto the substrate by an an insulin micro-syringe. The static contact angle of crosslinked and non-crosslinked scaffolds was measured by a digital camera (Fujifilm Fine Pix S 200EXR) in ambient conditions at room temperature (25 °C). The images of the water droplet images on the surface were captured and analyzed using Image J software (3).

5. Isolation of hUC-MSCs from umbilical cord Wharton's Jelly, Cell culture, differentiation, and MTT assay

MSCs were isolated from the umbilical cords of normal full-term infants. The umbilical cords were delivered to the laboratory in normal saline and stored at 4 °C until processing. The surface of the cord was rinsed with sterile phosphate buffered saline to remove as much blood as possible. Then, the umbilical cord was cut into 1 cm pieces. Wharton's jelly was isolated from the umbilical cords after the longitudinal section of each piece and removal of the umbilical cord blood vessels. Pieces of Wharton's jelly were seeded into a T-25 flask with DMEM containing 20% FBS, 5% Pen/Strep and, incubated at 37 °C, 5% CO_2. The culture medium was changed every 3 days until the mesenchymal stem cells reached approximately 80% (4).

MSCs from passages (P) 3 were used for the seeding into PVA/GE nanofibrous scaffolds. Before to cell seeding, scaffolds were sterilized by being immersed overnight in the 70% ethanol and ultraviolet light for 1 h per side, then they were placed into 24-well cell culture plates and PBS was added. The plates were then incubated at 37 °C, 5% CO_2, for 3 h. MSCs were seeded into the scaffolds with a cell density of 2×10^4 cells/well and kept in DMEM culture medium supplemented with 10% FBS for 2 days. Osteogenic differentiation medium (DMEM-F12 supplemented with 10% FBS, 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/mL ascorbic acid) was then added for 21 days. The medium was changed every two days. Osteogenic differentiation was confirmed by Alizarin Red S (2% in distilled water) staining (5). The viability of stem cells was determined by 3-[4, 5-Dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT method). This standard test method is based on the activity of a mitochondrial dehydrogenized of living cells that transform light yellow MTT into purple formazan product. The optical density (OD) corresponds to the viable cell numbers that were cultured on the crosslinked scaffolds and control samples (The control group was cell seeded over the cell culture plate). After being sterile, crosslinked PVA/GE scaffolds were placed in a 24-well culture plate and seeded with a cell density of 2×10^4 cells/well and maintained at 37 °C, 5% CO_2 under 200μl DMEM with 20% FBS. During the test, the culture medium was replaced every 2 days. After 3, 5, and 8 days of cell seeding respectively, the medium culture was replaced with PBS solution (0.01 M, pH 7.4) for washing the dead cells on the scaffold, and DMEM containing 10% MTT solution (with concentration level 5 mg/ml) was freshly added to

each culture well and the cultures were incubated at 37 °C and 5% CO_2 for 4h. The supernatant was removed, and 100µl of DMSO was added to each well. After 10 min of incubation, the OD values of the solution were measured at the wavelength of 570 nm using an ELISA reader (6). Each experiment was carried out in triplicate. The results were expressed as means ± SD. The absorbance values were compared using ANOVA and Tukey's test ($p < 0.05$). Besides, the cell viability was estimated from relationship 1:

$$Cell\ viability(\%) = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \qquad (1)$$

6. Degradation measurements and swelling test of PVA/GE nanofibrous scaffold

An important factor in tissue engineering is the measurement of scaffold degradation rate in a specific biological environment. PVA/GE nanofibrous scaffolds with dimensions of 1×1 cm was cut and sterilized by immersing in ethanol (70%) overnight and exposed to UV light for 1h per side. Dry scaffold samples were weighted (denoted as W_0) and placed in sample bottles containing 10 mL phosphate buffer saline (PBS) in pH 7.4. The scaffolds were incubated *in vitro* at 37 °C for 40 days. Every 5 days, three samples of scaffolds were removed from the bottles for characterization. After any given time, the samples were removed from the PBS solution, and the pH the solution was measured by pH meter. Then, the sample surfaces were wiped with filter paper and weighed (denoted as W_a). Afterward, each sample was washed with deionized water to remove any soluble inorganic salt and dried at room temperature to constant weight (denoted as W_t). Then, the water absorption percentage (W_A%) and weight loss percentage (W_L%) of scaffolds were calculated (relationships 2 and 3):

$$W_A\% = (W_a - W_0)/W_0 \times 100 \qquad (2)$$

$$W_L\% = (W_t - W_0)/W_0 \times 100 \qquad (3)$$

The prepared crosslinked PVA/GE scaffolds were immersed in PBS at pH 7.4. The scaffolds were incubated *in vitro* at 37 °C for 40 days. Every 5 days, three samples of scaffolds were removed from the 24-well plate and were wiped up with filter paper for characterization, and then swelling ratio (S%) was calculated (equation 4):

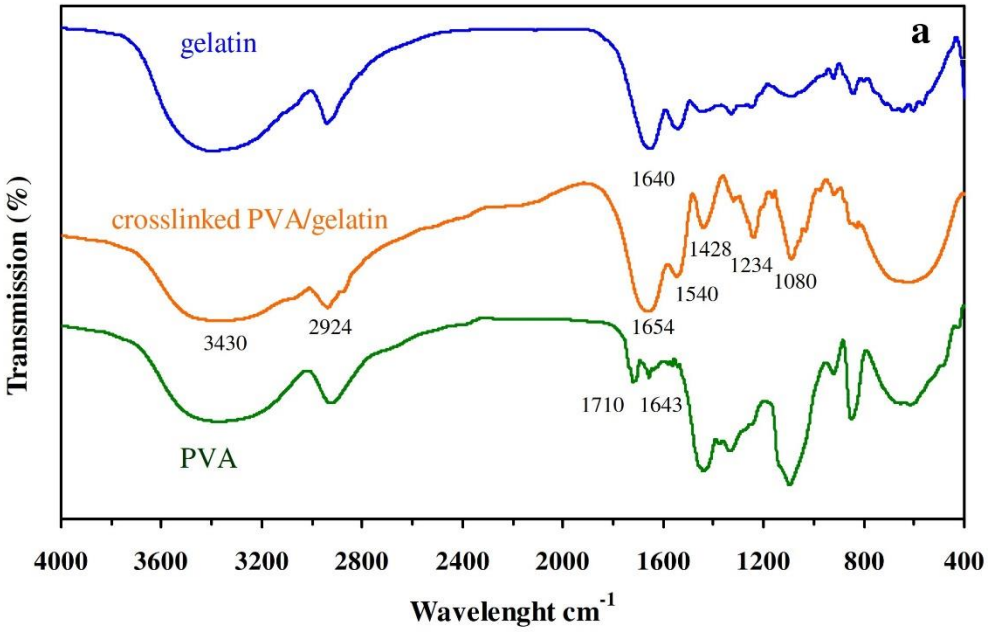
$$S\% = (W_w - W_d)/W_d \times 100 \qquad (4)$$

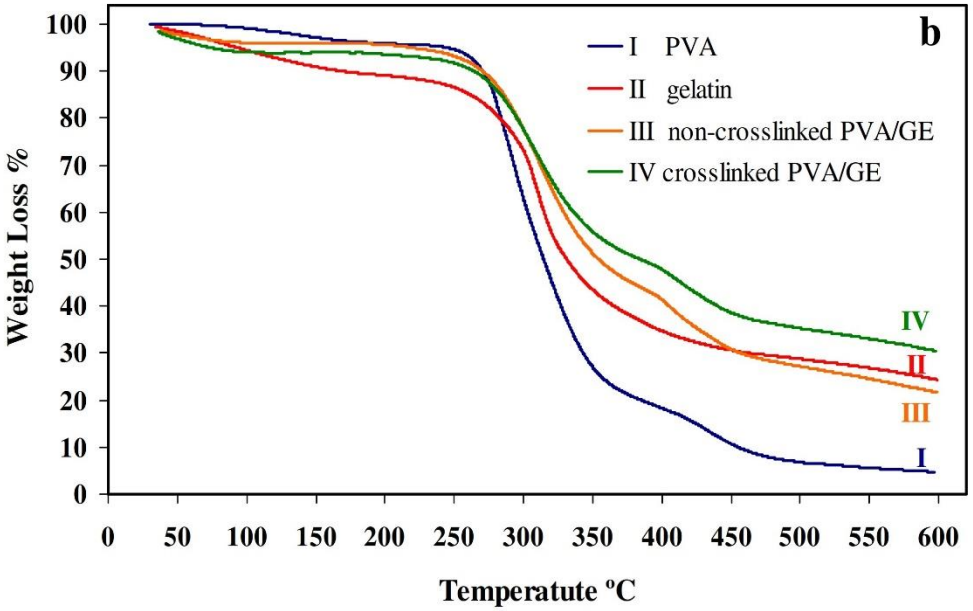
where W_w and W_d are the swollen scaffold mass and initial dried scaffold mass respectively.

7. FT-IR and TGA analysis

The functional groups and chemical structures of the PVA, gelatin, and crosslinked PVA/GE fibrous scaffolds were investigated by FT-IR spectra (Appendix 1a). The broad peak near 3380 cm^{-1} (in the range of 3000-3700 cm^{-1}) was observed in all the samples, which can be related to the stretching of inter molecular hydrogen bonds between hydroxyl groups of polymeric chains. As seen in FT-IR spectra of crosslinked PVA/gelatin, the reaction between PVA and gelatin with glutaraldehyde caused a decrease intensity of this peak compared with those of pure gelatin and PVA polymers because of acetal and imine formation. This is because O–H groups of PVA polymer and N–H and O–H groups of gelatins participate in crosslinking. In the FT-IR spectrum of PVA, the absorption peaks at 1710 cm^{-1} were attributed to the C=O band of residual acetates in the polymer chain and, the peak at 1080 cm^{-1} corresponded to C–O–C group. The distinct peaks at 1640 cm^{-1} in the gelatin FT-IR spectrum was ascribed to the stretching vibration of C=O in the polymer chain. Also, gelatin had N–H bending vibration, and C–H stretch at 1540 cm^{-1} and C–N stretch bending at 1234 cm^{-1}. During the crosslinking of PVA/gelatin with GA, the amino groups of gelatin and hydroxyl groups of PVA react with the aldehyde functional group of the crosslinker and form the aldimine (CH=N) and acetal linkages, respectively. The characteristic peaks of PVA, gelatin, and additional aldimine and acetal groups were observed in the FT-IR spectrum of crosslinked PVA/gelatin scaffold (7). Thermal properties of the PVA, gelatin, crosslinked PVA/GE, and non-crosslinked PVA/GE nanocomposites were studied using TGA curves (Appendix1b). The mass loss of PVA was taken place in three stages. The initial 5% weight loss was occurred between the temperature ranges of 30-180 °C and attributed to the evaporation of absorbed solvents and moisture on the polymer. The second region with about 75% weight loss at 240-400 °C was related to the thermal scission of PVA backbone due to dehydration of polymeric chain and formation of carbonyl compounds. The third stage, with about 14% weight loss around 400–600 °C was attributed to the ultimate destruction of carbonyl compounds. The thermal weight loss of gelatin was taken place in two stages; the first step with about 12% loss of initial weight at 30-200 °C was due to the removal of adsorbed solvents. The second stage with about 65% weight loss around 240-600 °C was attributed to thermal degradation of the polymeric chain. There are three weight loss stages in the TGA curve of the non-crosslinked PVA\GE scaffold. The second degradation stage of the non-crosslinked PV/GE

occurred at a higher temperature compared to the TGA curves of pure PVA and gelatin. It could be related to the interaction between the PVA and gelatin molecules that caused good compatibility between the two polymeric chains to enhance the thermal stability of the PVA\GE scaffold. Also, thermal stability of crosslinked PVA\GE scaffold was higher than non-crosslinked. It should be because of the formation of intense amide, Schiff's base, and ester bonds among GA, PVA, and gelatin molecules during chemically crosslinking (8).





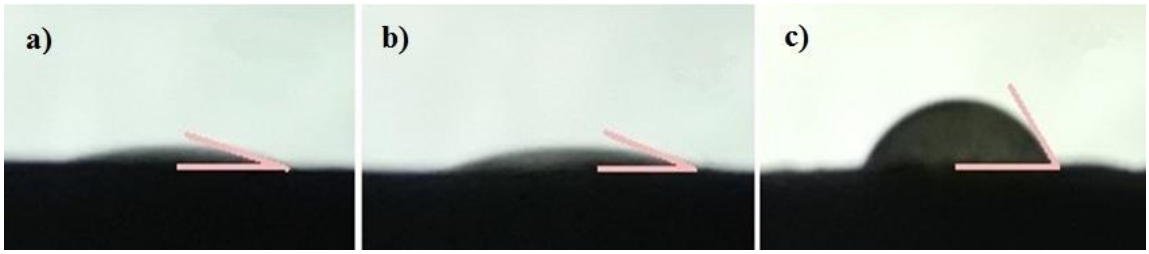
Appendix 1. (A) FT-IR spectra and (B) TGA curves of PVA, gelatin, and PVA/GE scaffold.

8. Contact angle measurement

The wettability studies of scaffolds investigating the contact angle of crosslinked and non-crosslinked scaffolds indicated that the contact angle of non-crosslinked PVA/GE scaffold was 14.7±1.2°. After treatment, the contact angle of scaffolds increased from 14.7° to 54.8° (Appendix 2). These results showed that hydrophobicity of nanofibers was increased (Appendix 3 a-c).

Appendix 2. Contact angle of scaffolds

Scaffold status	**Contact angle (θ)**
Non-crosslinked	14.7° ±1.2
Crosslinked with vapor of GA	18.1° ±2.1
Crosslinked with GA/methanol solution	54.8° ±1.8



Appendix 3. The contact angle of the (a) non-crosslinked, (b) crosslinked with vapor of GA, (C) crosslinked with GA/methanol solution, PVA/GE scaffolds.

References

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