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Development of biocomposite tissue scaffolds of collagen/gelatin/boron-doped bioactive glass prepared through solvent casting/particulate leaching method for bone tissue engineering

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One of the most important issues of modern medicine is the functional and aesthetic reconstruction of bone tissue after any loss. Selection of biomaterials and manufacturing techniques used in the production of tissue scaffolds is very important for bone tissue regeneration capacity. In this study, biocomposite tissue scaffolds were developed using collagen, gelatin, and boron-doped bioactive glass (B-BG). First, to increase bone formation capacity, bioactive glass was modified with boron and synthesized via sol-gel method. Then, the biocomposite tissue scaffolds were constructed through solvent casting/particulate leaching method by combining the B-BG with collagen/gelatin hybrid polymer solution and right after that the samples were cross-linked by using glutaraldehyde (GA) vapor. The structures of the scaffolds were characterized by scanning electron microscopy (SEM), X-ray diffraction analysis (XRD), and Fourier infrared transformation spectroscopy (FTIR), while hydroxyapatite (HA) accumulation was examined by using simulated body fluid (SBF). Besides, *in vitro* swelling capacity and degradation of the scaffolds were also investigated. Finally, *in vitro* cytotoxicity of the tissue scaffolds was evaluated using human osteoblast-like cell line (SaOS-2). All the results of tests revealed that the biocomposite tissue scaffold containing collagen/gelatin/B-BG can be a promising biomaterial to support the healing process of bone defects.

Keywords: Collagen, gelatin, boron-doped bioactive glass, composite tissue scaffolds, bone tissue regeneration.

Introduction

Bone is a collagenous composite connective tissue with both inorganic (60–70%) and organic (30–40%) parts/components. While most of the organic part in its structure is Type I collagen, the inorganic part contains calcium phosphate, which is mostly in hydroxyapatite (HA) form[1]. Millions of patients experience tissue loss every year due to reasons such as accidents, trauma, diseases, or surgeries. Bone tissue engineering focuses on developing biomaterials to enhance regeneration of bone tissue and restore its functions. Collagen, the main component of the skin and bone, is the most abundant element of the extracellular matrix and constitutes about 30% of the total mammalian protein mass. Collagen can be isolated from the tissues of mammalian animals and marine sources. Collagen-based biomaterials can be used in regeneration of several soft and hard tissues such as cornea, skin, bone, cartilage, lung, trachea, intestine, kidney, and uterus[2]. In particular, the use of collagen as a scaffold material in bone and cartilage tissue engineering is widespread since it acts as a template for calcium phosphate and calcium carbonate accumulation[3]. To reduce cost of the tissue scaffold production, some other natural polymers can be used together with collagen (i.e. gelatin). Gelatin is obtained by denaturation of collagen extracted from skin or bones of animals. Gelatin-based biomaterials provide appropriate medium for cell attachment and proliferation thanks to Arg-Gly-Asp sequence (RGD) in their structure[4]. Especially in the field of bone tissue engineering, collagen is combined with various bioactive inorganic materials and used as a composite system[3]. Bioactive glasses are suitable options as scaffold material for bone tissue engineering due to their osteoconductive and osteoinductive properties, and controllable biodegradation rates. They release bioactive ions that enable the stem cells transform into other bone cells via the osteogenic differentiation[5]. Metal ions (i.e. boron) can be added to bioactive glass structures during the

production process. Boron doped bioactive glass enhances the osteogenic differentiation of mesenchymal stem cells and proliferation of osteoblasts. Thanks to positive effects of boron on calcium metabolism, demineralization of bone is prevented, and bone mass is preserved by this way[6]. Bioactive glasses produced by the sol-gel method are promising materials that can ensure transport of boron to the target tissue to increase its regeneration ability.

The aim of this study was to produce B-BG containing collagen-gelatin scaffolds to obtain high bioactivity. The effect of the bioactive glass on the swelling capacity, degradation profile, cytotoxicity, and bioactivity of the scaffolds were investigated.

Experimental – Materials and methods

Materials:

Sodium tetraborate decahydrate ($Na_2B_4O_7.10H_2O$), sodium silicate ($Na_2O.3SiO_2$), calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$), sodium phosphate dibasic dihydrate ($NaH_2PO_4.2H_2O$), sodium chloride, 1 *M* nitric acid (HNO_3), and gelatin powder were purchased from Sigma-Aldrich. Acetic acid and 50 wt.% glutaraldehyde solution (GA) were purchased from Merck Inc.

Fabrication of B-BG particles:

The B-BG in SiO_2-CaO-Na_2O-P_2O_5-B_2O_3 system was produced by sol-gel technique. Production was carried out on a magnetic stirrer at room temperature. First, $Na_2B_4O_7.10H_2O$ was prepared in distilled water at 3% (w/w) concentration, $Na_2O.3SiO_2$ was added in ratio of 1:10 v/v into it. Afterwards, 2 *M* $Ca(NO_3)_2.4H_2O$ and 0.57 *M* $NaH_2PO_4.2H_2O$ were fully dissolved in 1 *M* HNO_3. This acidic solution was slowly dropped onto the basic solution. The solution was left for aging at room temperature for 24 h. After aging, the solution was centrifuged for 10 min with 8500 rpm. The solution was centrifuged for 3 times and B-BG was washed. The supernatant was removed, and B-BG phase was dried at 105°C for 24 h and calcined at 600°C for 2 h. Characterization of the B-BG was made by atomic absorption spectroscopy (AAS), Brunner-Emmett-Teller (BET) surface area analysis, thermogravimetric and differential thermal analysis (TG-DTA), XRD, FT-IR, and SEM analyses.

Preparation of tissue scaffolds:

First, 1 wt.% collagen solution was prepared in 0.5 *M* acetic acid under magnetic stirring at +4°C. Gelatin solution (2.5 wt.%) was prepared in distilled water. Hybrid polymer solutions were prepared to the ratio of gelatin to collagen by volume was 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, and 0:100. B-BG particles were added in 50 wt.% ratio to each sample. After homogeneous stirring, 80 wt.% NaCl particles were added to each sample. Amounts of B-BG and NaCl were based on the total dry weight of collagen and gelatin in each mixture. The mixtures were poured into molds (diameter~26 mm, height~21 mm), and frozen at –20°C. Then, lyophilization was carried out for 2 days at –52°C and 0.002 mbar conditions. After lyophilization, the scaffolds were crosslinked with 25 wt.% GA vapors in a desiccator for 24 h[7]. To remove GA residues as well as NaCl in the structures, the tissue scaffolds were first kept in 0.04 *M* glycine solution for 1 h, and then washing was carried out with 70% ethanol solution. After the washing process, the tissue scaffolds were left to dry in a desiccator for 5 days and stored at +4°C for characterization tests.

Characterization of the B-BG particles:

BET analysis of B-BG:

Surface area and pore structure of the B-BG were determined by BET analysis. The specific surface area, pore volume, and pore size of the B-BG particles were determined by Micromeritics TriStar II 3020 Version 3.02 Specific Surface Area and Pore Distribution Meter. Before analysis, the samples were subjected to degassing process under vacuum at 300°C for 16 h.

XRD analysis of B-BG:

Crystal structure of the synthesized B-BG particles was examined by X-ray diffractometer. Powder samples were analysed with the PANalytical brand X'pert PRO XRD device at a speed of 2 deg/min in the range of 5–90 degrees. Step size and scan step time were 0.0350 and 1 s, respectively.

FT-IR analysis:

The characteristic functional groups in the B-BG particles were analysed using FT-IR (Shimadzu-IRPrestige-21) spectroscopy and characterized with the transmittance values determined at a wavenumber of 4000–640 cm^{-1}. The transmission spectra of the samples before and after the calcination were measured to determine the impurities removed.

Characterization of the scaffolds:

Swelling capacity and degradation tests:

To study the swelling kinetics of the produced tissue scaf-

folds, small amounts (~10 mg) were taken from each scaffold. Each sample was immersed in 10 mL phosphate buffer saline (PBS, 0.05 *M*) with pH 7.4, and the samples were incubated at 37°C for 1 day. The dry weights of the scaffolds were determined before immersion, and the wet weights of the scaffolds were measured after 1, 2, 3, and 24 h incubation periods. The swelling capacity (%) of the tissue scaffolds after each wet measurement was calculated using the eq. (1), where W_S is percent swelling capacity, W_w is the wet weight of the tissue scaffolds, and Wd_1 is the dry weight of the tissue scaffolds. After 24 h swelling, the samples were dried at 37°C for 1 day and weighed (Wd_2). Degradation (%) of each sample was calculated using the eq. (2)[8].

$$W_S\ (\%) = [(W_w - Wd_1)/Wd_1]\times 100 \quad (1)$$

$$\text{Degradation}\ (\%) = [(Wd_1 - Wd_2)/Wd_1]\times 100 \quad (2)$$

Scaffold mineralization:

Simulated body fluid (SBF) was prepared based on the studies of Kokubo *et al.*[9]. Each scaffold was immersed in SBF for 1 and 7 days. At the end of the day 1 and day 7, characterization studies were carried out by SEM/EDS, XRD, and FT-IR. Swelling capacities (%) and degradation (%) of the samples were also calculated.

FT-IR analysis of the composite scaffolds:

The chemical bonds formed in the structures of the tissue scaffolds before and after SBF (day 1 and 7) were characterized by FT-IR.

XRD analysis of the composite scaffolds:

Characterization by XRD was performed to determine the crystal structure of biocomposite tissue scaffolds and to detect the presence of HA. Powder sample (sample with 50:50 ratio after 7 days SBF treatment) was analysed in the range of 10–60 degrees with a speed of 2 deg/min. Step size and scan step time were 0.02° and 1s, respectively.

SEM/EDS analysis of the composite scaffolds:

The microstructures of the scaffolds before and after SBF treatment were examined at different magnifications (250X-500X), and the elemental composition of the tissue scaffolds were analysed with EDS (Energy Dispersive X-Ray Spectroscopy) technique.

MTT assay of the composite scaffolds:

SaOS-2 cell line was seeded at 1×10^5 cells/mL into 96 well and kept in an CO_2 incubator at 37°C for 24 h. At the end of 24 h, the elution liquid obtained from the sample was added to the wells in three repetitions in concentrations of 1, 1/2, 1/4, and 1/8. The sample was left to interact with the cells in the wells for 24 h and then the MTT viability test was performed. 1% phenol solution was used as positive control and only DMEM medium (Dulbecco's Modified Eagle Medium) was used as negative control. Results calculated as the negative control was considered 100% alive. The absorbance values were measured at 570 nm with an ELISA (Enzyme-Linked Immunosorbent Assay) reader.

Results and discussion

Characterization of the B-BG particles:

The results of AAS analysis revealed that composition of the B-BG was 48.7% SiO_2, 19.2% CaO, 2.9% Na_2O, 21.3% P_2O_5, and 7.9% B_2O_3 in wt%.

According to results of BET analysis (Table 1), the B-BG particles have mesoporous structure with 5.7 nm pore size, also have high surface area with 161,4 m^2g^{-1}. The nitrogen adsorption isotherm was type IV based on the UIPAC classification which also indicates mesoporous structure.

Table 1. Textural properties of the B-BG particles

BET surface area ($m^2\ g^{-1}$)	Pore volume ($cm^3\ g^{-1}$)	Pore size (nm)
161	0,23	5,75

The result of TG-DTA analysis (Fig. 1) was used to determine calcination temperature. The B-BG particles has started to show more crystalline properties above 600°C. So, to increase the ion release and following apatite deposition on the B-BG surfaces in body fluids, calcination temperature was chosen as 600°C where the particles were more amorphous.

Some crystalline peaks were seen in the XRD pattern of the B-BG (Fig. 2), the sharp peak at $2\theta = 32.25°$ represents crystalline sodium calcium silicates.

SEM analysis (Fig. 3(a)) showed that the particles have angled, irregular, and non-spherical shapes. FT-IR spectra (Fig. 3(b)) was performed before and after the calcination. As a result of calcination process, carbon impurities were removed successfully.

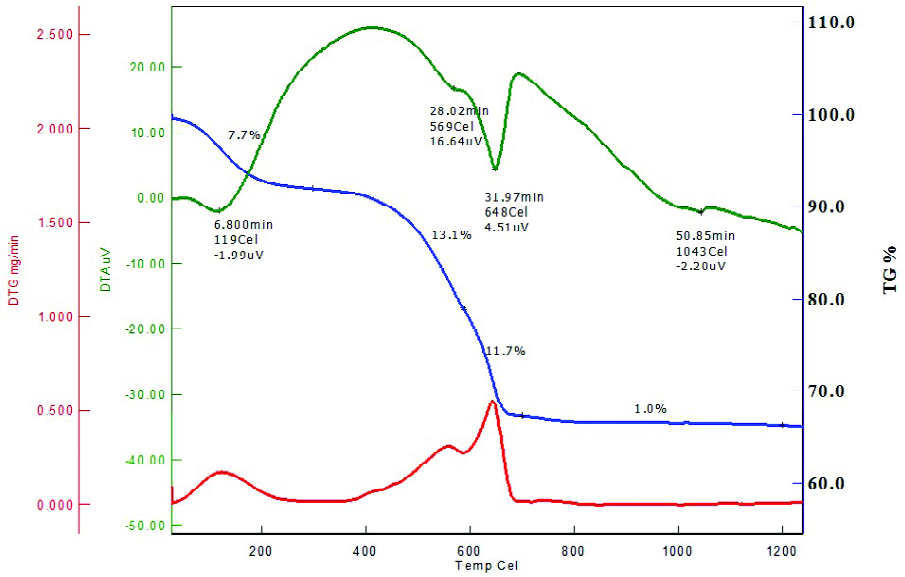

Fig. 1. TG-DTA graph of the B-BG particles.

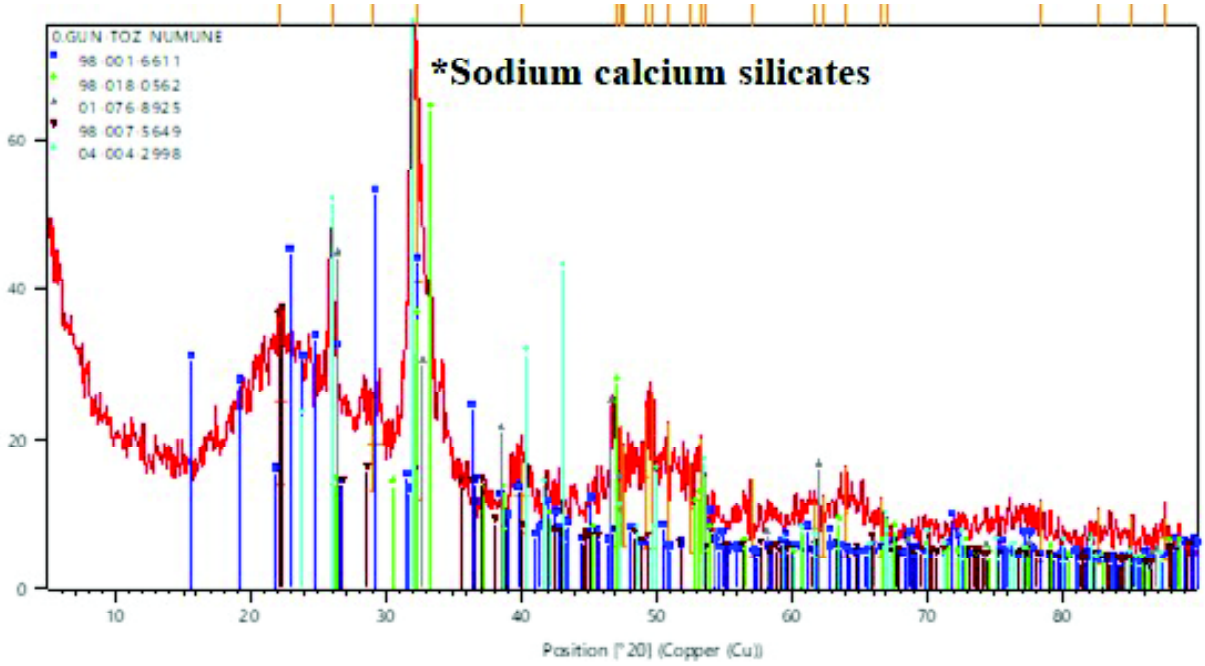

Fig. 2. XRD pattern of the B-BG particles.

Characterization of the scaffolds:

The results of swelling capacity and degradation tests indicated that the scaffold with 50:50 gelatin to collagen ratio (v/v) has the highest swelling capacity and the lowest degradation. Thus, 50:50 ratio was chosen as optimum for further characterizations. The diffraction pattern obtained from the sample with a 50:50 ratio after 7 days SBF treatment (Fig. 4) was compared with the standards set by the JCPDS (09-0432). Accordingly, the crystalline diffraction peaks of apatite were found mainly at the points where 2θ was 25.9º,

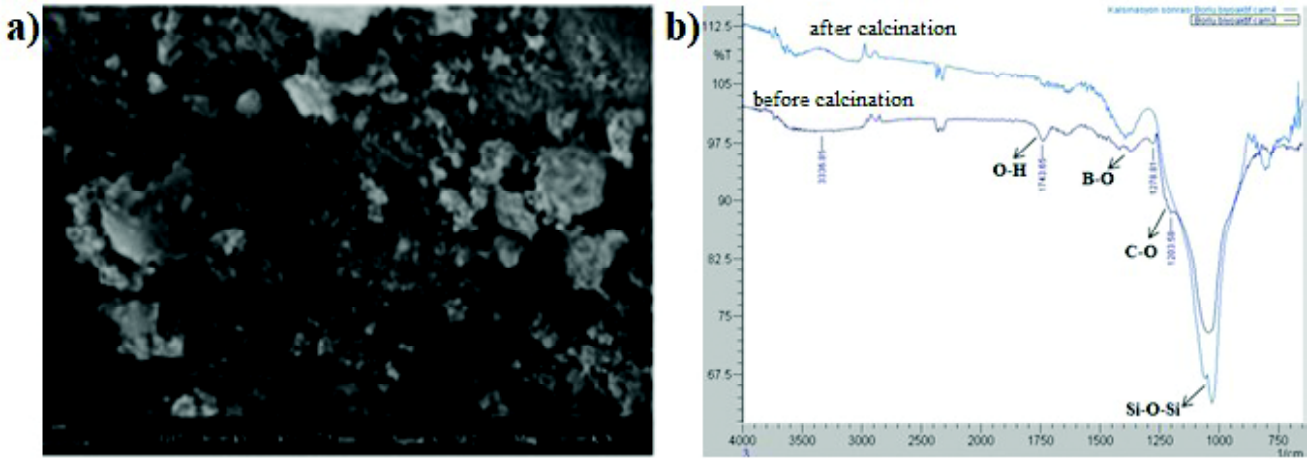

Fig. 3. (a) SEM images of B-BG particles at 10.00KX magnification; (b) FT-IR spectra before and after the calcination.

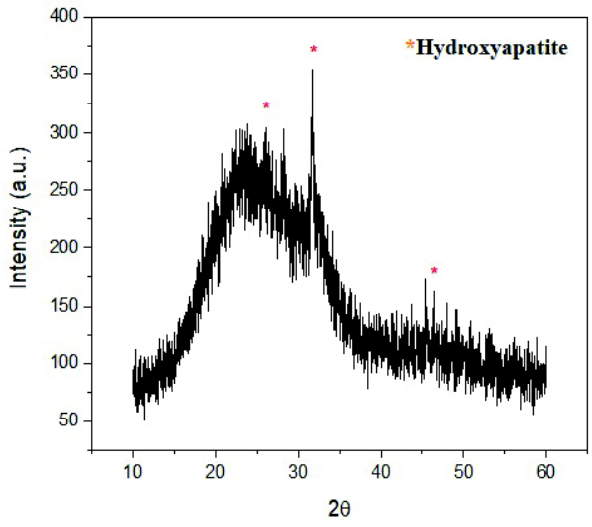

Fig. 4. XRD pattern of the scaffold after 7 days SBF treatment.

31.8°, and 46.7°, and these peaks are related to the (002), (211), and (222) planes, respectively.

In the SEM image of the scaffold with 50:50 ratio before SBF, the porous structure was clearly seen on Fig. 5(a). Average pore diameter was 127 μm which is suitable for bone ingrowth. The SEM-EDS results (Fig. 5(b)) revealed that the amount of calcium (Ca) and phosphorus (P) increased with increase of the immersion time in SBF. At the end of 7 days in SBF, the percentage of Ca and P ions were higher compared to day 0 and day 1, which indicates higher HA deposition on surface of the scaffolds.

FT-IR spectra of the scaffolds (Fig. 6) was obtained before and after day 1 and day 7 of SBF immersion. At day 7, it was observed that the peak expressing the P-O bond (970–1100 cm^{-1}) shifted and became a larger and sharper band

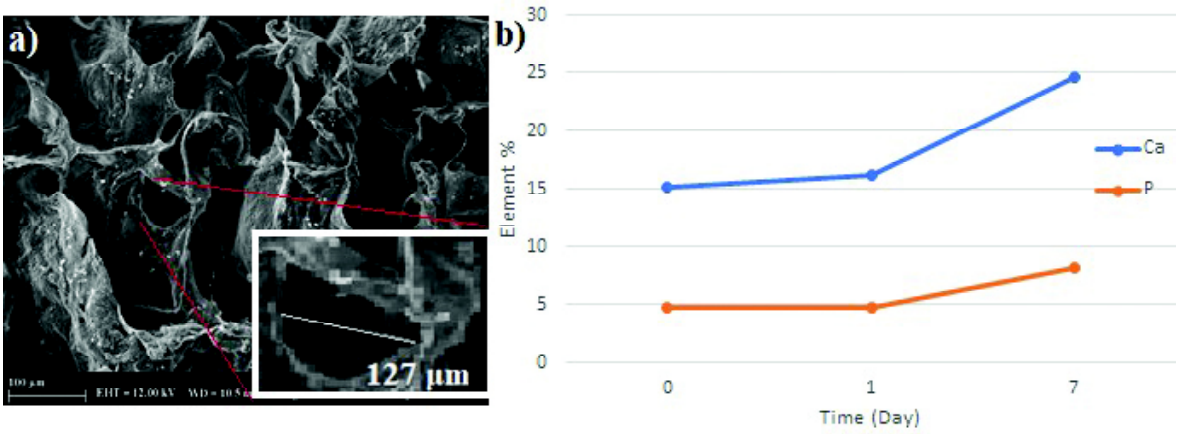

Fig. 5. (a) SEM image of the scaffold before SBF at 500X magnification; (b) graph of Ca and P amount at SBF days at 0, 1, and 7 days.

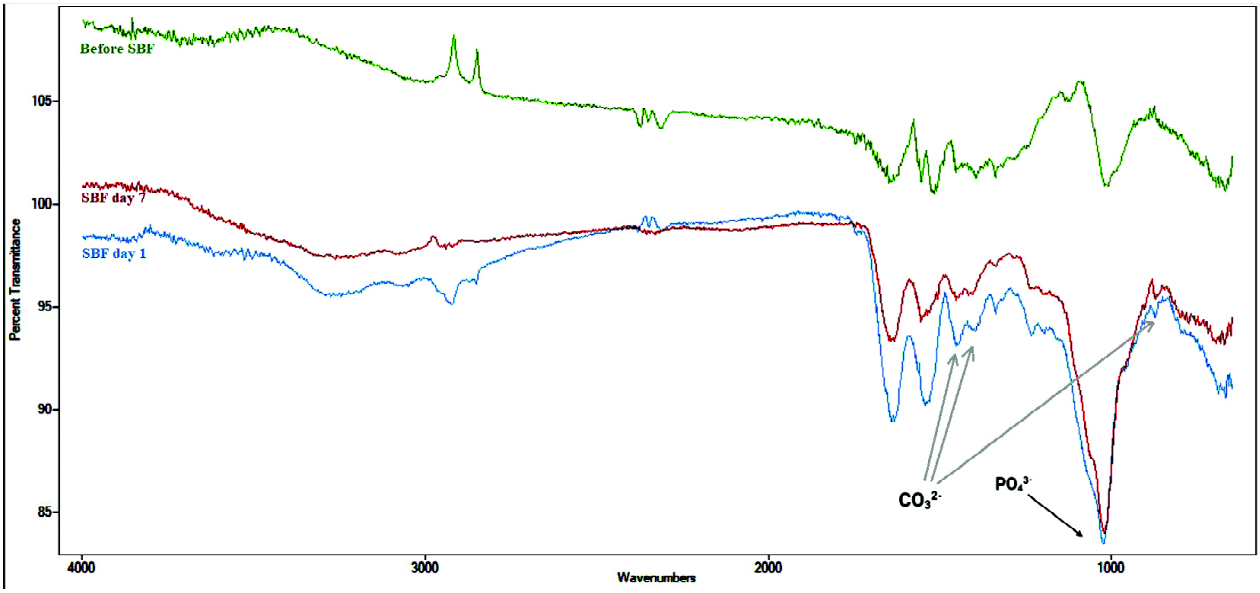

Fig. 6. FTIR spectra of the scaffold before SBF (green), day 1 (blue) and day 7 (red).

compared to the spectrum before SBF and SBF day 1. This means that there was a deposition of HA on the surface of the scaffolds by SBF treatment.

In vitro cell viability test:

The indirect MTT analysis (Fig. 7) on SaOS-2 cell line proved that the cells were not adversely affected by the scaffold, so their cellular metabolic activities were not damaged, and they continued to survive. In addition to this, it was proved that the toxic GA, which was used for crosslinking, removed successfully from the scaffolds as there was no cytotoxic effect on the cells.

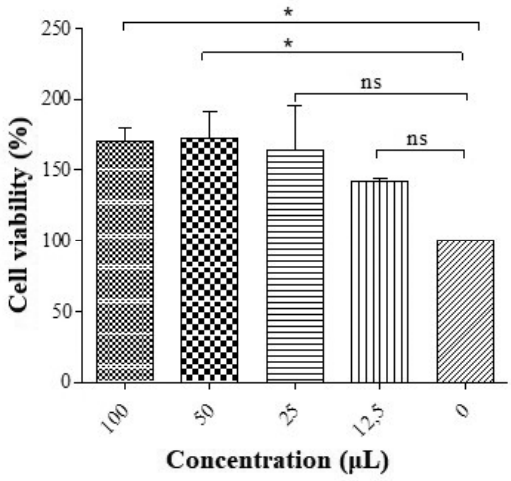

Fig. 7. Viability of SaOS-2 cells, Anova one-way analysis of variance was performed. ns: not significant, *p < 0.05.

Conclusions

In overall, the results indicated that the produced collagen/gelatin/B-BG biocomposite tissue scaffolds have special properties such as good osteoconductivity, porosity, and bioactivity that can enable their use in bone tissue regeneration. Based on the *in vitro* studies, to some extent, this research suggested synthesis of scaffolds using such polymers together with boron doped bioactive glass can mimic the chemical composition of native bone, induce bone formation, and provides new insights towards a potential treatment of diverse bone defects.

Acknowledgements

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