Fabrication and in-vitro evaluation of copper doped bioactive glass/polymer composite scaffolds for bone tissue engineering

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Research Article

Fabrication and in-vitro evaluation of copper doped bioactive glass/polymer composite scaffolds for bone tissue engineering

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Article Info	Abstract
	Composites developed by combining bioactive glasses and biopolymers are attractive materials for use in bone tissue engineering scaffolds due to their bioactivity, biocompatibility, osteoconductivity and mechanical properties. From this point of view, in this study, three-dimensional polymer/bioactive composite scaffolds were fabricated by using polymer foam replication method. To be able to achieve this goal, in the first stage new bioactive glass composition in the system SiO_2-CaO-Na_2O-P_2O_5 were developed with the incorporation of copper which have antibacterial and angiogenic properties. Scaffolds that mimic the structure of the foams were obtained after the heat treatment process. Then, the scaffolds were coated with gelatine at different percentages (1 and 3 weight%) in order to improve mechanical properties of the scaffolds. Microstructural, physical, chemical and mechanical properties of the composite scaffolds were investigated by using scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDS), Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction analysis (XRD), compressive strength test and porosity measurements. Furthermore, bioactivity and biodegradability behavior of the samples were determined by in vitro simulated body fluid (SBF) studies. The results showed that all scaffolds favored precipitation of calcium phosphate layer when they were soaked in SBF; they can also deliver controlled doses of copper toward the SBF medium. It was concluded that scaffolds coated with gelatine may be promising candidates for bone tissue engineering applications due to their porosity, bioactivity and appropriate biodegradation rate.

1. Introduction

Tissue engineering is an alternative approach to traditional methods for regeneration, repair and replacement of tissues and organs damaged as a result of trauma, infections or aging with the aid of scaffolds which have the ability to mimic the structure and function of the native tissues. Scaffolds that optimize cell integration with surrounding environment, cell migration and nutrient diffusion between the cells distributed within the matrix and the surroundings, provide a temporary framework for cells in order to constitute their own extracellular matrix (ECM) and degrade with the concurrent new tissue formation [1-7].

By virtue of innovation in bone tissue engineering, design of a novel scaffold with well-defined architecture is essential to fulfil the requirements in this field. The suitable scaffold

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should (i) be bioactive, biocompatible and biodegradable which encourages cell proliferation; (ii) has a highly porous three-dimensional (3-D) architecture with an interconnected pore network; (iii) has sufficient mechanical properties comparable to the host tissues permitting cell mechanoregulation to occur and structural integrity to remain [7-9]. Pore size and overall porosity are thought critical factors influencing bone tissue growth and nutrient transportation. However, mechanical strength is inversely related to increasing porosity of the scaffold. Therefore, the design of a suitable scaffold with optimum porosity and mechanical strength is a challenging issue [9-10].

Porous 3-D scaffolds were developed through utilizing a variety of materials including metals, polymers, ceramics and composites. Natural bone framework is a composite composed of organic components (25%) consisting mainly of collagen I and inorganic components (65%) consisting mainly of hydroxyapatite. The composite scaffolds as hybrid organic/inorganic biomaterials have hold great promise to mimic the natural bone composition [2].

Bioactive glasses comprise of a silicate network integrating sodium, calcium and phosphorus in various relative amounts are of great interest in biomedical applications due to their high bioactivity. However, low mechanical properties of bioactive glasses restrict their use in load-bearing applications. Bioactive glass/biopolymer composite materials that manipulate the flexibility of polymers with the stiffness and bioactive property of the bioactive glasses have enhanced mechanical properties, chemical stability or biological reactivity [11]. Common methods for fabrication of bioactive glasses include traditional melt quenching method and sol-gel method [12,13]. The foam replication method used in scaffold fabrication draws attention because of its ability to controlling pore size and distribution, not using toxic chemicals and simply adjusting the structure of the foam template [14].

The composition of porous bioactive glasses widely accepted in the systems of SiO_2-CaO or SiO_2-CaO-P_2O_5, has been improved with controlled amounts of therapeutic ions such as Cu^{2+}, Co^{2+}, Sr^{2+} and Ag^+ due to their antibacterial activity, osteogenic and angiogenic properties. Wu et. al. [15] explained the facility of released copper ions to induce osteogenic and angiogenic response and promote bone regeneration. In this study, Cu-doped bioactive glass/polymer composite scaffolds were fabricated by using polymer replication method.

2. Materials and Methods

2.1. Preparation of Bioactive Glass

Bioactive glasses in the system of SiO_2-Na_2O-P_2O_5-CaO-CuO containing 0.5% copper oxide by weight were produced by conventional melt-quenching technique. The nominal composition of this glass is, in weight% 45 SiO_2, 24.5 Na_2O, 6 P_2O_5, 24 CaO and 0.5 CuO. For this purpose, appropriate amounts of silicon dioxide (SiO_2, Sigma-Aldrich), di-sodium hydrogen phosphate (Na_2HPO_4, Merck), calcium carbonate ($CaCO_3$, Merck), copper (II) nitrate ($Cu(NO_3)$, Sigma-Aldrich) and sodium carbonate (Na_2CO_3, Merck) were first placed in a platinum crucible. The mixture was melted at 1623 K for 1 h and then quenched into deionized water. As-prepared glasses were ground and kept at 1623 K for 2 h in order to reduce the viscosity of the glass. The obtained bioactive glasses were ground (≤45μm) for obtaining homogeneous structure.

2.2. Scaffold Fabrication

Scaffolds were prepared by using a polymer foam replication technique. Firstly, the appropriate amount of polyvinyl alcohol (PVA) was dissolved in deionized water for 1 h at 343 K. Once the homogeneous solution was obtained, the temperature was reduced to 313

K and glass powder was dispersed in the solution under constant stirring. The composition of as-prepared solution is 6.28% PVA, 52.37% water and 41.35% glass powders. Polyurethane foams (60 PPI, pore per inch-152 pores/cm) which were cut into 10 x 10 x 10 mm samples, were immersed in the prepared solution for 2 min in order that the foams were coated with bioactive glass particles. Afterwards, as-coated foams were dried at room temperature overnight and subjected to a controlled heat treatment process for 1.5 h at 1223 K and for 2 h at 823 K to remove the polymer and sinter the glass. The scaffolds were then, immersed in the 1wt% and 3 wt% gelatine (50% Type A and 50% Type B) solutions for 2 min. The coated scaffolds with gelatine were left to dry at room temperature overnight.

2.3. Structural Analysis

FTIR spectra were collected using a Perkin-Elmer, Spectrum 100 Model spectrometer in transmittance mode in the mid infrared region (650-4000 cm^{-1}) for determination of chemical structure of scaffolds before and after immersion in simulated body fluid (SBF). X-ray diffraction (XRD) patterns of coated and uncoated scaffolds were recorded using PANanalytical X'Pert Pro diffractometer to determine the characteristic phases and amorphous structure of the scaffolds. Samples were ground and measured in powder form for XRD and FTIR analysis.

2.4. Surface Morphology

Scanning electron microscopy with energy dispersive spectroscopy (SEM-EDS, JEOL 5410) was utilized for morphological and elemental analysis of coated and uncoated scaffolds. Prior to the SEM measurements, all of the samples were coated with platinum under vacuum for 120 seconds by using sputter coater (SC7620, Quorum Technologies Ltd) in order to reduce electron charging effects.

2.5. Assessment of Bioactivity

Biodegradability of coated and uncoated scaffolds and formation of bone-like apatite on various surfaces were evaluated in vitro through immersion of samples in SBF, as described by Kokubo et al. [16]. Each sample of dimensions $5 \times 5 \times 5$ mm^3 was immersed in 30 ml of SBF and was stored in an incubator at controlled temperature of 37 °C. Samples were immersed in SBF for different soaking periods: 1, 7, 14, and 28 days. When samples were removed from the SBF solution, they were rinsed with ethanol and water, and dried at 37 °C for 30 minutes. The samples were then characterized using SEM and XRD.

2.6. Water Uptake and Weight Loss Measurements

Water absorption (%W_A) and weight loss (%W_L) of samples upon immersion in SBF were determined over the 28-day period using equations (1) and (2). The initial weights (W_I) of the scaffolds were recorded before they were immersed in SBF. Afterwards, prepared samples were immersed in 30 ml of SBF and incubated at 310 K for 1, 4, 7, 14, 21 and 28 days separately. Samples removed from SBF were dried at room temperature for 1 h and then weighed (W_W) to measure water absorption (%W_A). Subsequently, samples were left in an incubator at 310 K overnight and weighed (W_D) to measure the weight loss (%W_L). Water absorption (%W_A) and weight loss (%W_L) of the samples were calculated using the Eqs. (1) - (2) respectively.

$$\%W_A = [\frac{W_W - W_D}{W_D}] \times 100 \tag{1}$$

$$\%W_L = [\frac{W_I - W_D}{W_I}] \times 100 \tag{2}$$

2.7. Mechanical Properties

The compressive strength of samples (dimensions: 5 × 5 × 10 mm³) was measured using a Shimadzu AGS-J servo-hydraulic testing instrument. The crosshead speed was 1 mm/min. At least five specimens for each sample series were tested. Average values and standard deviations were determined.

2.8. Porosity

Porosities of the coated and uncoated scaffolds were measured by using Quantachrome Poromaster Model porosimeter. Experimentally measured raw data were evaluated through a microcomputer data acquisition system and total pore volume was determined.

2.9. Copper Release Investigations

Release properties of scaffolds were investigated by measuring the changes in the concentration of copper in the SBF solution as a result of the soaking of scaffolds for predefined time steps (1, 4, 7, 14, 21 and 28 days) using inductively coupled plasma optical emission spectrometry (ICP-OES). A Perkin Elmer Model Optima 2100 ICP operated at 13.56 MHz (using Ar and N_2 gases) was used for the measurements.

3. Results and Discussions

3.1. Structural Analysis

FTIR analysis was performed to identify the functional groups in the samples. For this purpose, the absorbance values of coated and uncoated samples recorded at 650-4000 cm⁻¹ were examined. Figure 1 shows the transmittance spectra of uncoated and coated scaffolds. The characteristic absorption bands detected in the range 1000-1100 cm-1 were attributed to Si-O-Si bending stress and the characteristic absorption peak at 900 cm⁻¹ indicated the presence of Si-O-Si asymmetric stretching. FTIR results showed that regular tetrahedron SiO_2 structure is formed in the samples. Furthermore, the appearence of the broad peaks in the range of 2900-3000 cm⁻¹ are attributed to unsaturated asymmetric O-H stretching vibration due to –OH group related to moisture of the sample. Furthermore, it was determined that the absorption peak at 1600 cm⁻¹ in the spectra of samples coated with 1% and 3% gelatine can be assigned to the N-H bending vibration in the amine groups in gelatine [17,18]. This result showed that the scaffolds were coated with gelatine and gelatine was held on the surface successfully.

3.2. Microstructural Analysis

Figures 2, 3 and 4 show the SEM images and EDS results of uncoated and coated scaffolds respectively. It is observed in Figure 2 that scaffold fabricated by the polymer replication method has a three dimensional, open and interconnected macrostructure similar to that of the used polyurethane foam. Moreover, it is seen that the pore walls are completely formed and the thicknesses are in appropriate size that prevents the decay of the structure. Figure 3 indicates that gelatine has attached onto the scaffold surface homogeneously without blocking the macroporous structure. As seen in Figure 4, the pore structure of the sample coated with 3% gelatine is not completely formed, however gelatine coating covered the sample in a homogeneous manner. In addition, 3% gelatine solution causes some of the pores to become clogged. Furthermore, according to the EDS results, it has been observed that Ca^{2+}, Si^{4+}, P^{5+} and Na^+ ions forming the glass composition were present on the surface of the scaffolds. Since Cu^{2+} ion content in the glassy structure is too law, copper cannot be detected in the samples due to the measurement limit of the EDS instrument.

The porosities of the uncoated and coated (1% and 3% gelatine) scaffolds were measured as 81%, 62% and 55%, respectively. According to SEM analysis results and porosity measurements, it is concluded that scaffolds coated with 3% gelatine are not appropriate

for bone tissue regeneration. On the other hand, samples uncoated and coated with 1% gelatine may be suitable candidates for bone tissue regeneration due to higher porosity values than the assumed minimum porosity requirement of 50% [1]. The pore size distributions of uncoated and coated scaffolds are given in Figure 5.

3.3. Mechanical Behavior

The compressive strengths of the uncoated, 1% and 3% gelatine coated samples were measured as 0.052, 0.062 and 0.61 MPa, respectively. The mechanical strength of the scaffolds increased by coating with gelatine. Gelatine layer increases the mechanical strength by covering some pores and filling the micro cracks.
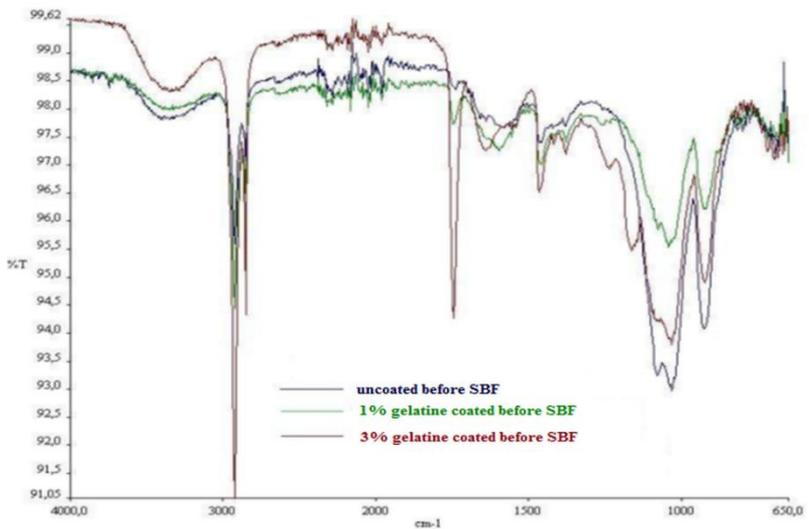

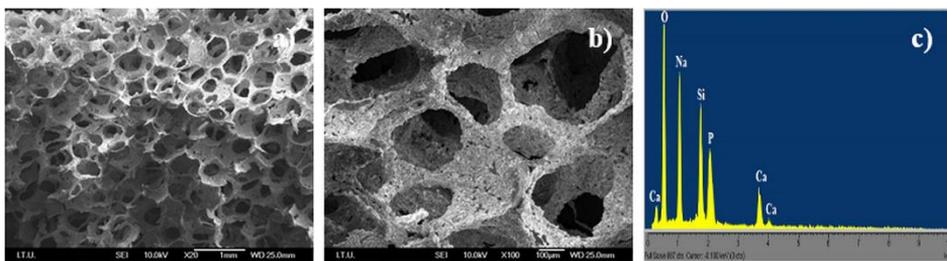Fig. 1 FTIR spectra of the scaffolds before immersing into SBF

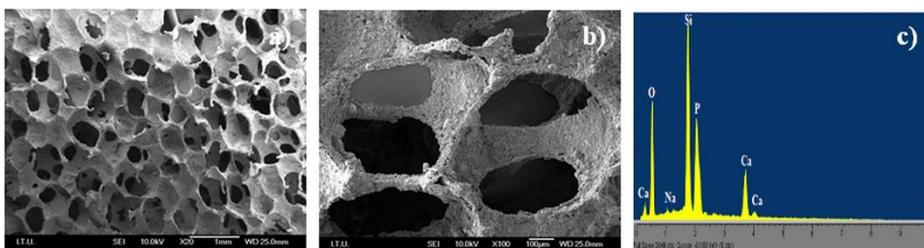Fig. 2 (a) and (b) SEM images of uncoated scaffold and (c) EDS result of the sample

Fig. 3 (a) and (b) SEM images of 1% gelatine coated scaffold and (c) EDS result of the sample
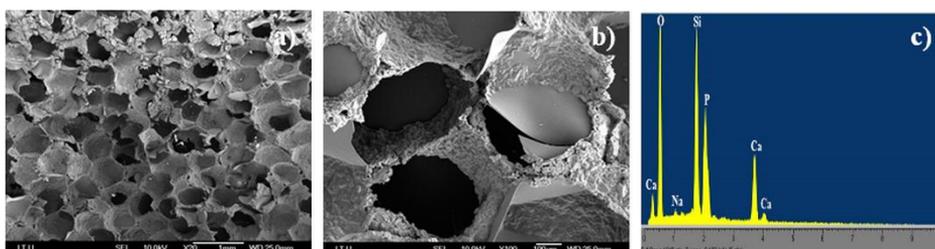

Fig. 4 (a) and (b) SEM images of 3% gelatine coated scaffold and (c) EDS result of the sample

3.4. Bioactivity Assessment

Surface modification of the produced scaffolds after contact with SBF was analyzed using SEM-EDS, XRD and FTIR analysis. Figures 6, 7 and 8 show the SEM images and EDS results of uncoated and coated scaffolds after 28 days immersion in SBF, respectively. It was observed that the hydroxyapatite layer was formed on the surface of the scaffolds from SEM micrographs. As a result of EDS analysis, Ca/P ratio for scaffolds uncoated, coated with 1% and 3% gelatine was determined as 1.53, 1.8 and 1.4, respectively. Ca/P ratio of the scaffold coated with 1% gelatine is closer to HA crystal structure with Ca/P ratio of 1.667. The formation of hydroxyapatite on the surfaces of scaffolds after immersion in SBF was confirmed both SEM and EDS analysis.

As seen in Figures 9, 10 and 11, peaks at 25°, 27°, 32° and 50° 2θ were detected in XRD patterns of uncoated and coated scaffolds immersed in SBF for different time steps. These peaks confirmed the formation of crystalline HA layer on the surface of the scaffolds after one-day contact with SBF. It has been also detected that coating scaffolds with gelatine improved the bioactive behaviour of the scaffolds due to the high bioactivity of the gelatine. According to the XRD results, it is concluded that crystalline HA layer become evident with the increase of soaking time in SBF. Scaffolds coated with 1% gelatine are highly bioactive materials.

FTIR spectra of uncoated and coated scaffolds after immersion in SBF for 28 days is given in Figure 12. P-O bonds, one of the most determinant indicators of formation of the HA layer could not be observed due to the minimum wavelength 650 cm^{-1} of the FTIR used in this study. After 28 days immersion in SBF, observed peaks at 875 cm^{-1} are attributed to carbonate hydroxyapatite (CHA).
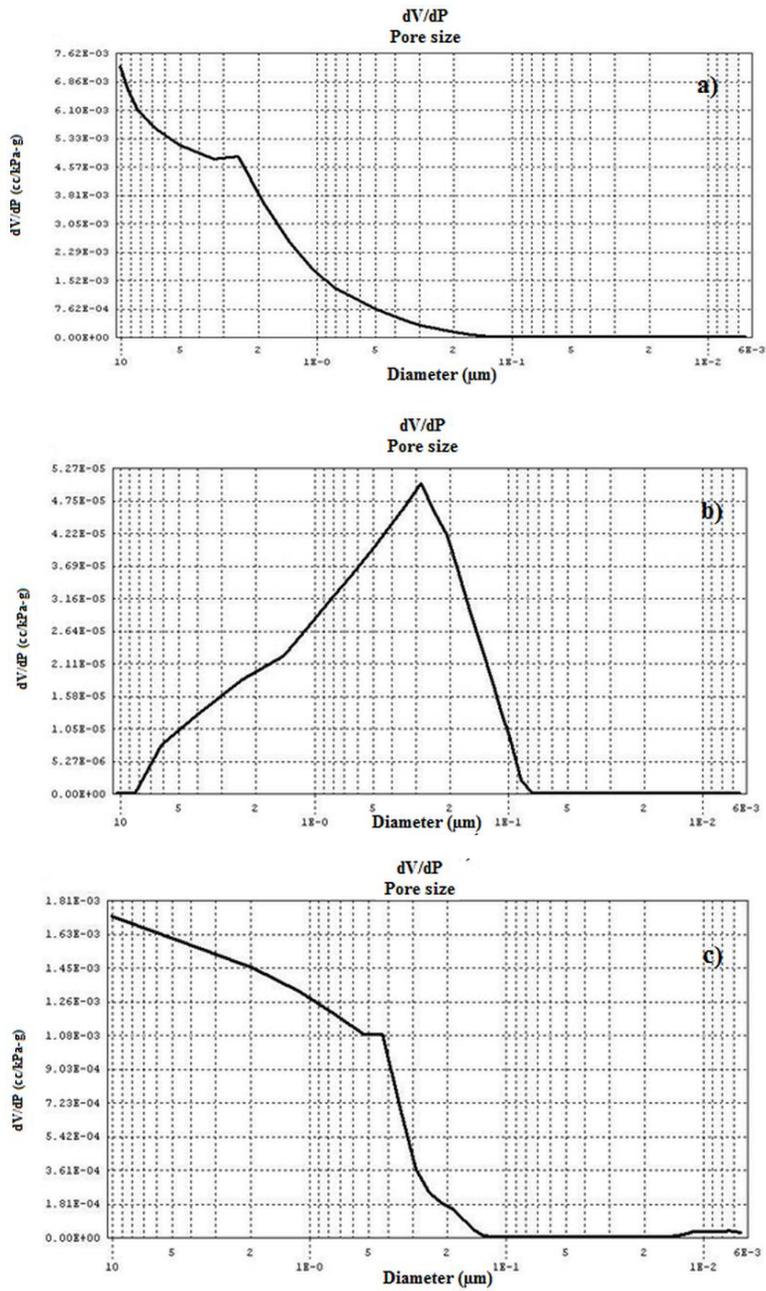
Fig. 5 Pore size distributions of scaffolds uncoated (a), coated with 1% (b) and coated with 3% (c) gelatine
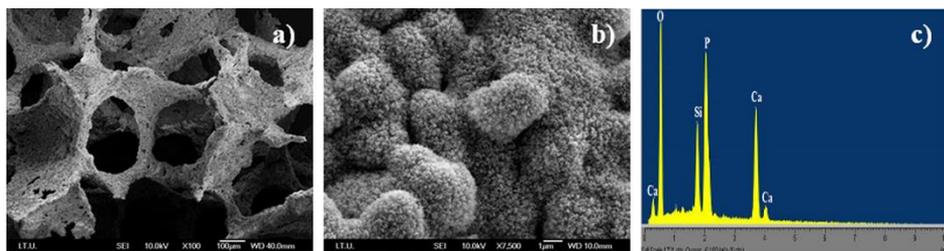
Fig. 6 (a) and (b) SEM images of uncoated scaffold after 28 days immersion in SBF and (c) EDS result of the sample
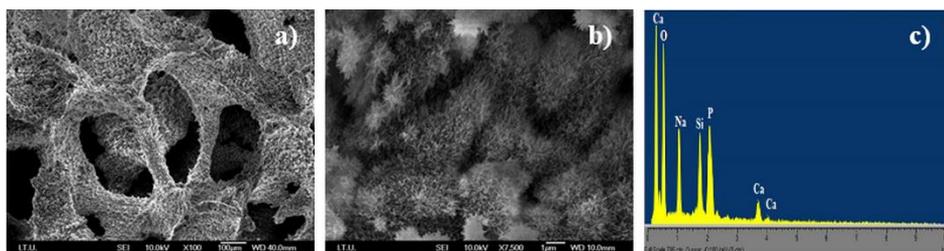

Fig. 7 (a) and (b) SEM images of 1% gelatine coated scaffold after 28 days immersion in SBF and (c) EDS result of the sample
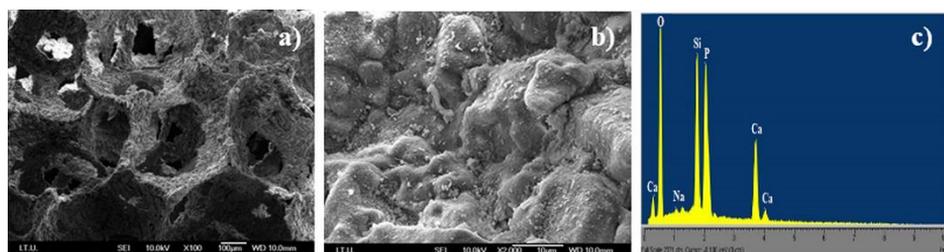

Fig. 8 (a) and (b) SEM images of 3% gelatine coated scaffold after 28 days immersion in SBF and (c) EDS result of the sample

3.5. Biodegradation Behavior

Water absorption and weight loss of scaffolds were investigated in order to determine biodegradability of the samples immersed in SBF for different time periods. According to water absorption analysis results given in Figure 13 (a), compared with the uncoated scaffolds, the rate of water absorption was increased in the coated scaffolds. As seen in the Figure 13 (b), weight loss analysis results showed that the rate of weight loss was increased with gelatine coating ratio. It is concluded that coated scaffolds have higher biodegradation rate.
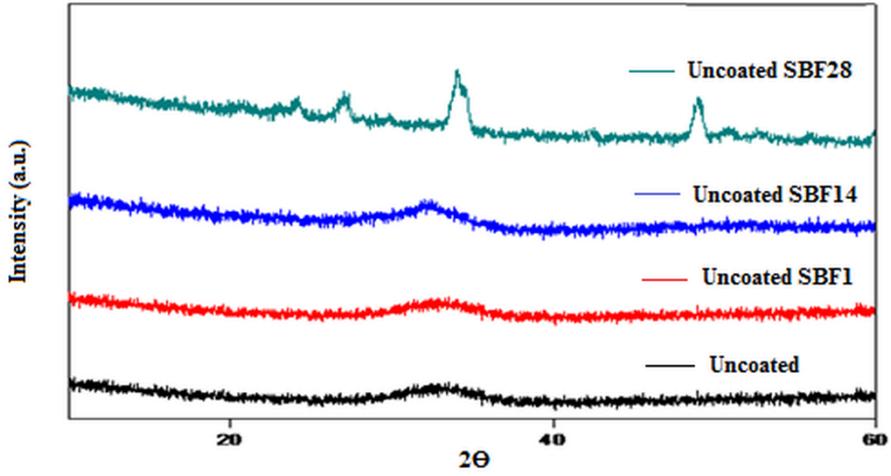
Fig. 9 XRD patterns of uncoated scaffolds before (a) and after (b) 1, (c) 14 and (d) 28 days immersion in SBF
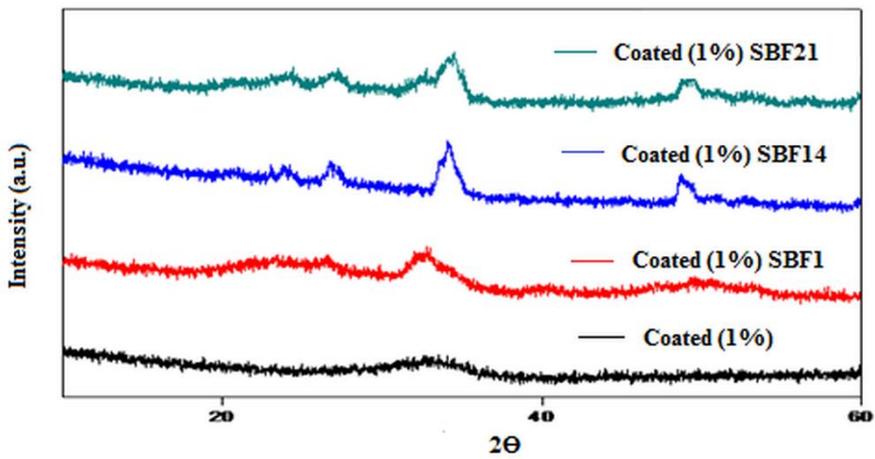

Fig. 10 XRD patterns of 1% gelatine coated scaffolds before (a) and after (b) 1, (c) 14 and (d) 21 days immersion in SBF
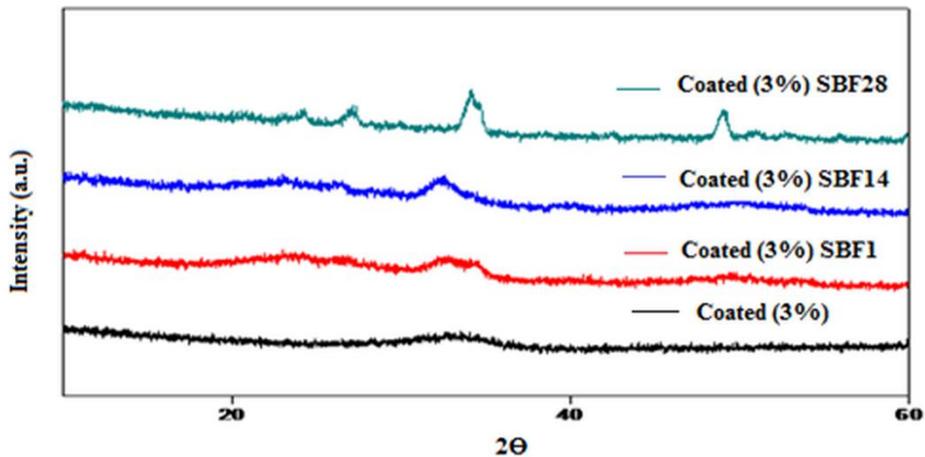
Fig. 11 XRD patterns of 3% gelatine coated scaffolds before (a) and after (b) 1, (c) 14 and (d) 28 days immersion in SBF
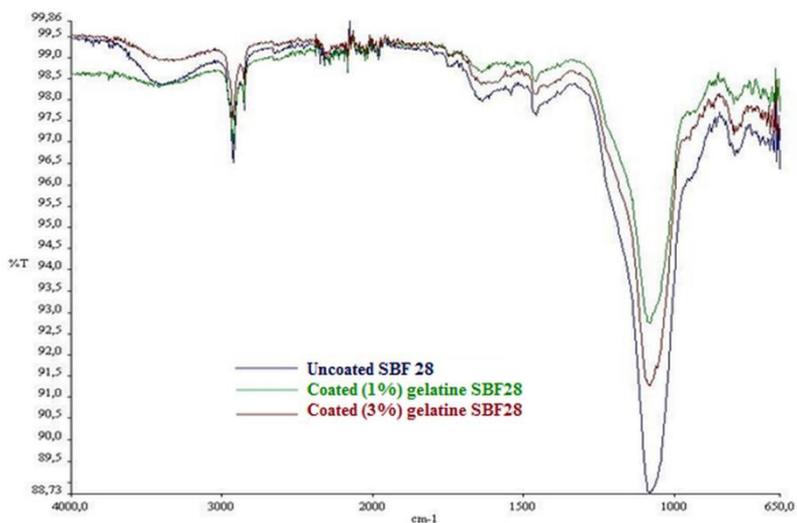

Fig. 12 FTIR spectra of scaffolds (a) uncoated (b) coated with 1% and (c) coated with 3% (c) gelatine after 28 days immersion in SBF
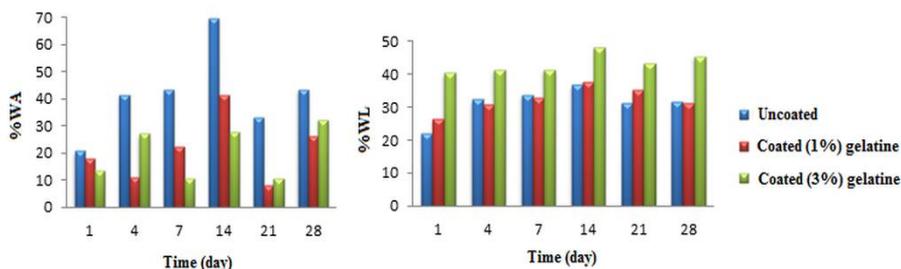
Fig. 13 (a) water absorption and (b) weight loss values for uncoated and coated scaffolds after immersion in SBF for 1, 4, 7, 14, 21 and 28 days

3.6. Copper Release

Copper release behaviour of scaffolds after immersion in SBF for different time periods were evaluated by ICP analysis. In Table 1, cumulative concentration of Cu^{2+} ions released from the uncoated and coated scaffolds into the SBF solution is given. It is clear that copper release from scaffolds increased with immersion time. Furthermore, Cu^{2+} ion release from uncoated scaffolds is higher than that of the release from coated samples because of the rate of glass dissolution process. It is reported that toxic level of copper concentration is accepted as 58 ppm. In addition, cell vascularization and rate of bone formation were increased with copper concentrations higher than 5.6 ppm [19]. From this point of view, it can be said that the obtained scaffolds have angiogenic properties without any toxic effects.

Table 1 Copper ion release from uncoated and coated scaffolds after immersion in SBF

Scaffolds	Copper concentration (ppm)					
	1 day	4 days	7 days	14 days	21 days	28 days
Uncoated	2.356	6.015	7.060	7.650	9.030	11.80
Coated (1%) gelatine	2.352	6.020	6.720	7.620	9.080	10.160
Coated (3%) gelatine	1.826	5.963	6.211	7.470	9.050	10.540

4. Conclusion

In the present study, 0.5% copper doped bioactive glass/gelatine composite scaffolds with interconnected macropores were successfully fabricated by using foam replication method. Porosity measurements and SEM analysis results indicated that produced scaffolds have suitable microstructure for bone tissue engineering requirements. It was found that microporous structure of scaffolds supports bone formation. Pore walls of the scaffolds are completely formed and the thicknesses are in appropriate size that prevents the decay of the structure; however, it was observed that coating with 3% gelatine solution causes some of the pores to become blocked. Uncoated samples and coated samples with 1% gelatine showed higher porosity values than the assumed minimum porosity requirement of bone. As a result of FTIR analysis, observed peaks related to Si-O-Si bonds which are the basic vibration bands of glassy structure and peaks attributed to N-H amine groups indicated that the scaffolds were coated with gelatine and gelatine was held on the surface successfully. According to EDS analysis, Ca/P ratio of the scaffold coated with 1% gelatine is determined as 1.8 which is closer to HA crystal structure with a Ca/P ratio of

1.667. In addition, presence of copper in the glass system promoted the effective bone regeneration. Mechanical strength of the scaffolds were improved by coating scaffolds with gelatine. All analysis proved that the scaffolds immersed in SBF had hydroxyapatite forming ability which is relevant to bone regeneration. The coated scaffolds exhibited improved bioactive behaviour. XRD patterns of uncoated and coated scaffolds immersed in SBF for different time intervals confirmed the formation of crystalline HA layer on the surfaces of the scaffolds. Furthermore, XRD analysis showed that after heating process and coating with gelatine, the amorphous structures of the samples were remained. This is the result of the fact that scaffolds improve the bioactivity properties. According to biodegradability studies, coated scaffolds showed enhanced biodegradability behaviour compared with the uncoated sample. Copper release studies indicated that produced scaffolds can release controlled doses of copper toward the SBF medium that is the determinant for bone tissue regeneration. Within this respect, copper doped bioactive glass/polymer scaffolds offer great perspectives and this results will be subject of further studies.

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