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Synthesis and Physicochemical Characterization of Biomimetic Scaffold of Gelatin/Zn-Incorporated 58S Bioactive Glass

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Abstract**—**The main purpose of this research was to design a biomimetic system by freeze-drying method for evaluating the effect of adding 5 and 10 mol. % of zinc (Zn) in 58S bioactive glass and gelatin (5ZnBG/G and 10ZnBG/G) in terms of structural and biological changes. The structural analyses of samples were performed by X-Ray Diffraction (XRD), scanning electron microscopy (SEM) and Fourier-transform infrared (FTIR) spectroscopy. Also, 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and alkaline phosphatase (ALP) activity tests were carried out for investigation of MC3T3-E1 cell behaviors. The SEM results demonstrated the spherical shape of the formed hydroxyapatite (HA) phases and also HA characteristic peaks were detected by XRD spectroscopy after 3 days of immersion in the simulated body fluid (SBF) solution. Meanwhile, FTIR spectra proved that the intensity of P–O peaks for 5ZnBG/G was more than 10ZnBG/G and control samples. Moreover, the results of ALP activity test illustrated that the optimal amount of Zn (5ZnBG/G) caused a considerable enhancement in bone cell growth. Taken together, the scaffold with 5 mol.% Zn was introduced as an optimal sample because of its higher biocompatibility, *in vitro* bioactivity and growth of MC3T3-E1 cells in comparison with other samples in bone tissue engineering.

Keywords**—**Scaffold, gelatin, modified bioactive glass, ALP, bone tissue engineering.

I. Introduction

BONE is a rigid organ that constitutes part of the skeleton in most vertebrate animals. Additionally, bone is a connective tissues that contains about 90% collagen [1]. Fortunately, bone is one type of tissues in the human body that can repair itself if damaged [2] but unfortunately if the bone is severely damaged, it cannot repair itself [3] though thanks to tissue engineering (TE) bone can return to its pre-injury state [1]. Methods like autografts [4], [5] or allografts [6] are limited due to problems such as tissue donation restrictions, the possibility of transmitting viruses [7] and stimulating immune system responses [8]. Therefore TE [9]-[11] was created as a solution to replace injured tissue with living tissue. One of its advantages is possibility of designing this tissue based on the requirements of each patient [12].

Scaffolds are a suitable selection in TE and for meeting the needs well they should have special characteristics such as biocompatibility [13], suitable chemical and physical properties, optimal degradability rate [14], appropriate porosity and appropriate mechanical property based on the target tissue [15], [16]. Among various kinds of materials, ceramics and polymers are not individually ideal candidate as scaffold in bone tissue engineering due to their brittleness and insufficient strength, respectively [17]. Therefore, composites which are mostly polymeric/ceramic [18] were introduced as the novel generation of these alternatives because of their special properties like appropriate strength [19]. These scaffolds contain a mineral phase like HA [20] or tricalcium phosphate (TCP) [21] along with collagen [22], chitosan [17], [23] and gelatin [24]. Gelatin is recognized as a biodegradable and biocompatible material [24] and causes an increase in the mechanical property of bioactive glass (BG) phase [25] that is derived from collagen with lower cost and easier process [25], [26]. In addition to biocompatibility, BGs have a successful bond with the body tissue when they are placed in body conditions by performing chemical reactions. After performing reactions in biological conditions, these glasses form a rich layer of HA, which is due to a strong connection at the interface between the glass and the tissue that is resistant and strong against the incoming stresses. BGs have a special place in TE due to their unique properties such as a homogeneous structure due to the use of special chemical compounds, the property of guiding bone growth along with the induction of bioactivity, and the quick connection of the possibility of controlling the range of chemical properties gained. Also, with the advancement of nanotechnology, nano-composites have been a suitable alternative for composites in recent years [27]. In fact, because of having freer surface, they have more mechanical conflict with the base material and will be stronger if chemical bonds form [28].

The gelatin-bio ceramics scaffolds are made by process that works with the penetration of calcium (Ca) ions and phosphate (P) ions into gel [29] and because of the presence of the middle gel like the primary cartilaginous structure in the body, they are more similar to bone formation in the body [30]. Finally, for preparing the porous scaffolds, because of cleanliness, simplicity and controllability for creating scaffolds with interconnected pores, freeze-drying method has been suggested [31], [32]. Creating this type of porosity is considered as an influential structure on the activity of cells and transferring them into the site of bone formation [33].

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In addition, providing biomimetic conditions, modifying the scaffolds by modifiers such as zinc (Zn) [34], magnesium (Mg) [35] and strontium (Sr) [32] have been suggested. Meanwhile, Zn, because of its main role in the synthesis of proteins in the body and growth of the skeletal system, is more significant compared to others [21]. Also, Zn has been known for many years as an effective element in bone formation [36]. Previous report has shown that Zn has two roles in bone formation, firstly encouraging osteoblast cell differentiation and secondly inhibiting osteoclast differentiation [36] in contrast, reducing bone density, delaying bone growth in animals and increasing the risk of osteoporosis in humans are effects of Zn deficiency [37]. Furthermore, it was found that Zn has an anti-inflammatory effect [38]. Thian et al. have shown that HA containing Zn had a more bioactivity property and led to an increase in the mesenchymal cells derived from human adipose tissue and production of the markers of bone cell [36] and presence of Zn prevented the growth of HA crystals [39]. Additionally, 1.04 mol.% of Zn in HA caused an increased differentiation and ALP activity of osteoblast cells [40].

Based on the above and previous studies about composites scaffolds by biomimetic method, it seems that the study of effect of different ions on the properties of scaffolds through penetration has great importance [41], [42]. Despite the recent researches about the effect of Zn in scaffolds properties, the optimal molar percentage of Zn in BG and gelatin phase has not been reported. The aim of this study is comprehensive investigation on the formation of BG phase inside the hydrogel structure by the biomimetic method and analyzing structural and *in vitro* biological properties of produced scaffolds in order to introduce an optimal composite scaffold. These analyses were performed by applying XRD, FTIR and SEM for structural evaluation and ALP activity and 3 MTT for biological investigation. In this study, a SBF solution was used for providing a similar condition to the human body and freeze-drying method for the production of porous scaffolds.

II. Materials and Methods

A. Required Materials and Synthesis of Specimens

In order to create specimens by biomimetic process, the amount of 10 wt. % gelatin solution was made. It was used powder of gelatin, and by adding it into distilled water at a considered temperature range from 47 °C to 57 °C, the solution was prepared. It should be noted that a stirrer was applied when gelatin powder was added. The amount of solution was considered according to the size of mold. After the preparation solution, at first, it was poured inside of mold in the middle section, and after that, it was kept in refrigerate for a duration of 1 day in order to hydrogel preparation. After hardening and removing the temporary section, other solutions were prepared. In this step, preparation of zinc nitrate ($Zn(NO_3)_2$), calcium chloride ($CaCl_2$), and phosphate salt with buffering properties (Na_2HPO_4) solutions was done. Also, the pH of solution was constant at 7.4 by using Tris (hyroxymethyl)aminomethane and hydraulic acid [20]. In the next step, $CaCl_2$ and Zn ions were poured on the same side of hydrogel, and Na_2HPO_4 solution was poured on the other side of mold. In the final step, the solution was put in refrigerate for 7 days. After this time, the solutions precipitated inside the gel, and composites were formed. The gelatin hydrogel was removed from the mold, and the composite part was cut. It should be noted that the incorporation of Zn in BG phase was selected in range of 0-10 mol.%. Additionally, the designed chemical compositions and schematic of procedure are shown in Table I and Fig. 1, respectively.

TABLE I
The Chemical Composite Scaffolds (Molarity)

Specimen	Calcium Molarity	Phosphor Molarity	Zinc Molarity
BG/G	0.1	0.06	-
5ZnBG/G	0.095	0.06	0.005
10ZnBG/G	0.09	0.06	0.01

For making a porous scaffold, freeze-drying method was applied. In this process, all specimens were put in the freezer for a duration of 24 h and then were placed in the freezer dryer (with specification VirTis Genesis-SP) for a duration of 24 h. Moreover, in the next step, all specimens were immersed in 1% glutaraldehyde solution in order to create crosslinking between chains of gelatin and enhanced the mechanical property. The immersion time was considered about 24 h. Finally, specimens were washed with water and alcohol, and the process of their drying was done by a freezer dryer. For evaluation of *in vitro* bioactivity of specimens, SBF was prepared. All the primary chemicals used were of analytical grade and were purchased from Merck Company.

B. Characterization of Specimens

1. Fourier-Transform Infrared (FTIR)

The aim of FTIR (USA 670, NEXUS) was: (i) To identify created groups in composites scaffolds, which were synthesized and produced by a biomimetic process; (ii) To evaluate effect of immersion time in SBF solution on bonds formation and their intensity. The FTIR spectra was recorded in the range of 400-4000 cm^{-1} wavenumber.

2. Scanning Electron Microscopy (SEM)

SEM (TESCAN) was implemented to monitor the morphology, structure of formed porosity, and size of pores in all scaffolds. It should be noted that for increasing the resolution of SEM's results, it was coated the surface of specimens with a layer of gold.

C. Investigation of Cell Behaviors of ZnBG/G Specimens

1. Culture of Osteoblast Cells (G292)

The culture process was done by using human osteosarcoma cells. For culturing, all cells, after finishing their defrosting, were put in a culture medium. This culture medium contained FBS (10%) RPMI. After that, the process of incubating was performed at temperature 37 °C. Finally, all cells were put in the culture medium, which contained scaffolds, and their culture process was done for a duration of 7 days. After the mentioned steps, cell morphology was analyzed.

2. 3-(4, 5dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT)

Cells should be incubed for 24 h before starting the MTT test. This step was done due to measuring the cell's adhesion. The volume and quantity of cells were considered 100 µl and 10^4 per well. In the next step, the extract, which was taken from scaffolds, was added to the medium and all system was incubated for a duration of 24 h. After removing the culture medium, the MTT solution (100 µl) was added. Also, removing the MTT solution was performed after 4h, and 100 µl of isopropanol was poured into the medium. Finally, the concentration of material, which was dissolved in isopropanol, was measured.

3. Evaluation of ALP Activity

For measuring the ALP activity, at first, 10,000 cells were put on the scaffolds, and then culture medium (100 µl) was added. In the next step, 1 ml of culture medium was added and after 3 h, a substance was ready for ALP activity investigation. Again after 24 h the culture medium was added (1 ml), and finally, the ALP activity was analyzed after 3 h, after collecting the culture medium from specimens.

4. Statistical Analysis

All experiments were performed at least three times. The obtained results were evaluated by Graph Pad Prism software (V.8.0, Graph Pad Software, and USA). It should be noted that all results were reported by mean ± standard deviation (SD), and probability value (*p) (*p < 0.05) was considered as statistically considerable.

III. Results and Discussion

A. FTIR

Fig. 1 shows the obtained results after immersion in the SBF solution. It was seen that the peaks of phosphate were formed in all specimens in the wavenumber 500 (between 500 and 600), and 1030 cm^{-1}. Also, the intensity of peaks increased by enhancing the time of immersion. To be more precise, it was a sign of increasing formed phosphate in the structure, and it could be found that all specimens showed good bioactivity property after placement in the SBF solution. In addition, another new peak was observed in the region of 860 and 3450 cm^{-1}, which was for O-H groups. It was because of the replacement of CO_3^{2-} groups instead of OH^- in the HA structure [46].

Also, the presence of CO_3^{2-} groups might be due to the absorption of carbonate from the environment, which in both cases can be a reason for the formation of carbonate apatite in the scaffolds after immersion in the SBF solution [43]-[45]. It should be noted that the presence of Zn ion led to increasing the intensity of peaks in comparison with the control specimens while the 5ZnBG/G was more influential. The intensity of PO peaks for 5ZnBG/G was more than 10ZnBG/G. Moreover, previous studies reported that substituted Zn in calcium phosphate bioceramic led to decreasing the intensity of OH and other peaks in FTRI spectra results [469, [47]. In fact, because of the ability of 5ZnBG/G in the formation of HA and also according to the XRD results, this specimen exhibited better *in vitro* bioactivity in comparison with 10ZnBG/G specimen and was chosen for SEM evaluations.

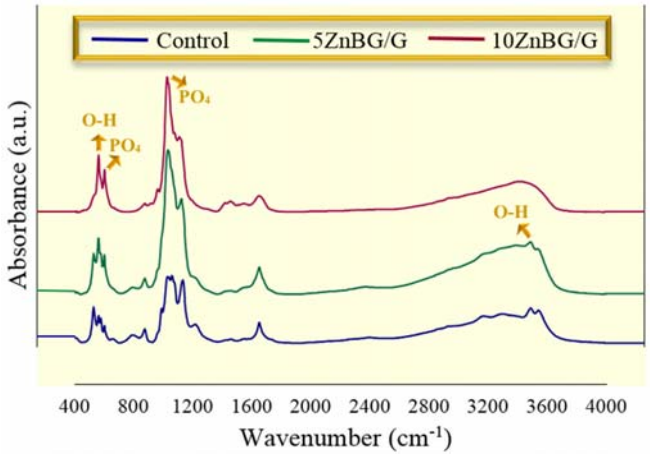

Fig. 1 The FTIR spectra of all specimens after 14 days of immersion in SBF solution

B. SEM Analyses

The SEM images for 5ZnBG/G before and after immersion in SBF are presented in Fig. 2. According to SEM images for this specimen, before soaking in SBF solution, sediment particles were scattered on the surface and inside the pores of scaffolds. The shape of these particles was almost spherical, and their size was about 100 nm, which is shown in Fig. 2 (A). Moreover, as defined in composites, spherical reinforcing particles have a significant effect on increasing the toughness of the composite [48].

In the SEM images related to 5ZnBG/G specimen, which was immersed in the SBF solution. In Fig. 2 (B), the crystals of sediment particles were observed. Morphologically, these particles had a smooth surface and were needle-shaped. As could be seen from the obtained results, the formed crystals had a more specific surface area. Therefore, it leads to creating a higher contact surface to react with ions in the extracellular matrix and bone cells [49]. It should be noted that by comparing the results of the optimal specimen before and after soaking in the SBF solution, it could be concluded that the amount of sediments formed has increased significantly because of high bioactivity of synthesized specimen. In other words, high bioactivity of this scaffold led to more formation of HA on the surface. As it was reported previously, substituted Zn in collagen scaffolds led to form needle-shape precipitates [34]. Moreover, the morphology and size of porosity are shown in Fig. 3. It was observed that the shape of the holes was a honeycomb structure, and the holes were interconnected, which can transfer oxygen and food. In fact, these properties create better conditions for the growth of cells. Also, the size of porosities was in the range between 200 and 500 microns; as previously reported, this range is the optimal size for bone cell growth [6], [12]. It should be noted that 5ZnBG/G specimen had the optimal size between 200 and 500 microns.

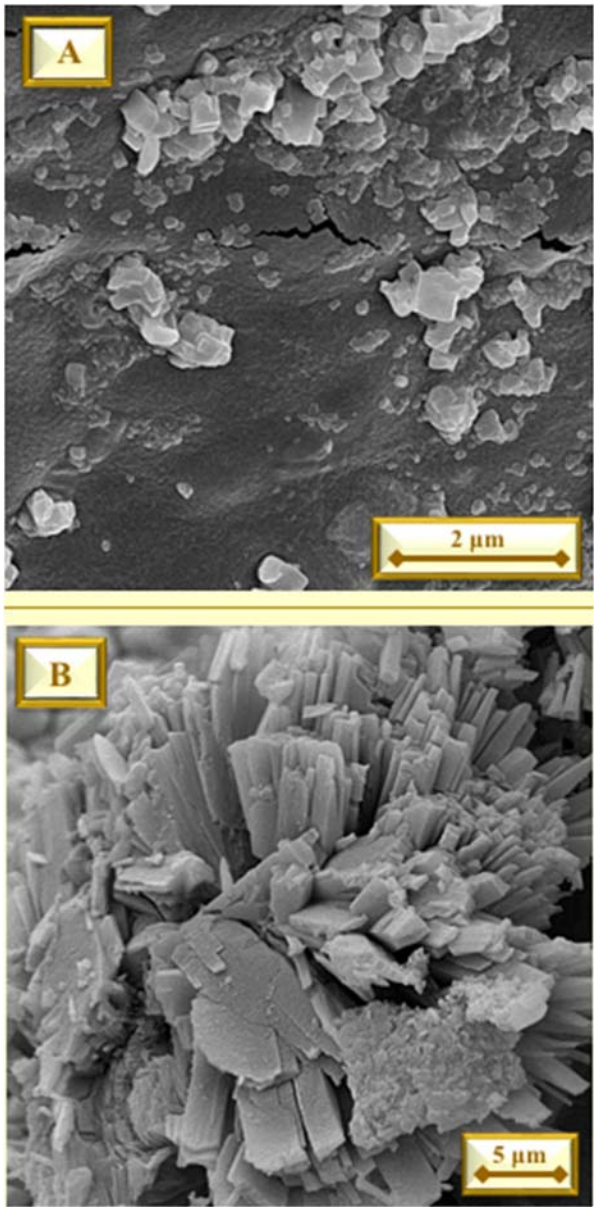

Fig. 2 The SEM images results 5ZnBG/G before (A) and after (B) immersion in SBF solution

C. In vitro Bioactivity of Composite Scaffolds

1. MTT

Fig. 4 presents the results for MTT assay by measuring the amount of OD. The results were analyzed for 1 and 7 days after culturing process. As it was clear, 5ZnBG/G was the only specimen that caused stimulate cell proliferation. The obtained amount of OD for this specimen was 9% more in comparison with the control specimen (**p < 0.01). Moreover, 10ZnBG/G showed a toxicity effect compared to the control specimen (*p < 0.001). In fact, for this specimen, OD decreased after 1 and 7 days at the rate of 8% and 18%, respectively. It can be found that the kind of composition and also the time of culturing have affected the MTT results. Because, on the one hand, substituted 5 mol. % Zn was influential about the function of calls, however, 10 mol. % had a toxicity effect. On the other hand, as it was observed for 10ZnBG/G specimen, this toxicity effect increased after 7 days immersion (**p < 0.01). Additionally, it has been reported that substituted Zn in HA caused an increase in the human cells (adipose-derived MSCs) growth. It should be noted that a low amount of Zn (1.6 wt. %) was used [50]. Another investigation revealed that Zn has a dose-dependent manner, and scaffolds containing Zn did not show a detrimental effect on cell activity. Also, it was reported that the high amount of Zn did not have a considerably metabolic effect with the increasing time of *in vitro* experimental [34]. As a result, based on the obtained data, 5ZnBG/G specimens were considered more effective specimen for cell growth.

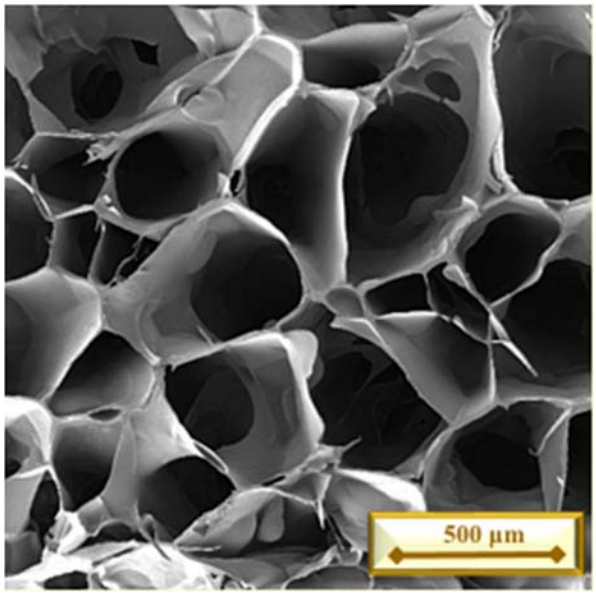

Fig. 3 The morphology result 5ZnBG/G specimen

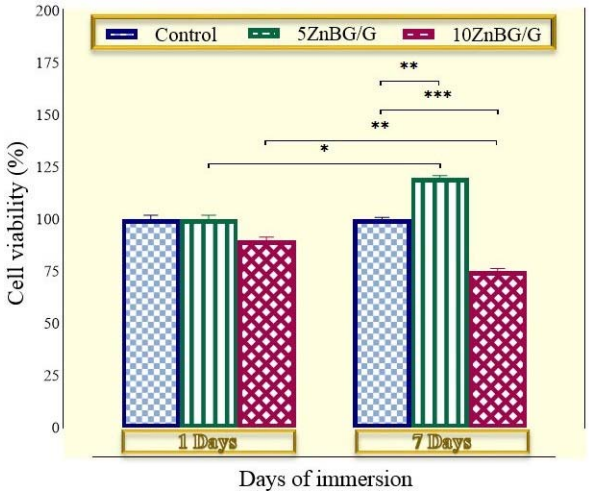

Fig. 4 The MTT results of all specimens after 1 and 7 days culturing period

2. ALP

Fig. 5 illustrates the results of ALP activity of specimens after co-culturing with the human osteosarcoma cells (G292). The results showed the enhancing effect of 5ZnBG/G on ALP

activity compared to other specimens. ALP activity increased in 5ZnBG/G specimen at the rate of 0.56 after 3 days. Moreover, adding 5 mol. % Zn in scaffolds compositions caused to create significant ALP activity compared to the control specimen (*p < 0.01). While, 10ZnBG/G had a reverse effect, and the ALP activity decreased considerably in comparison with the control specimen (**p < 0.001). As it is clearly shown, significant differences between 10ZnBG/G and 5ZnBG/G were observed. Because, with the decreasing amount of Zn from 10 to 5 mol. % the amount of ALP activity increased about 29% (**p < 0.001). Additionally, a previous study investigated that Zn can increase the ALP activity in a low amount in the culture medium of SaOS-2 human osteoblastlike cells [51]. Moreover, it has been presented that the presence of the low amount of Zn (2 mol. %) in the structure of HA increased the ALP activity of osteosarcoma cells compared to pure apatite [52]. Therefore, the 5ZnBG/G was considered as an optimal composite scaffold in terms of ALP activity.

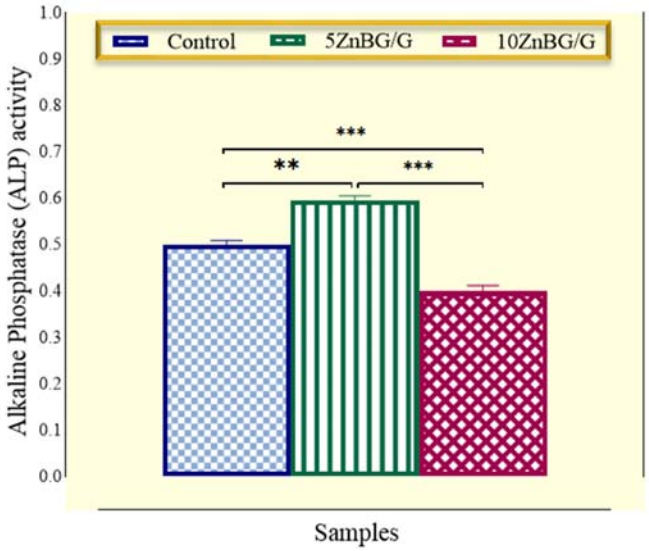

Fig. 5 The ALP activity results of all specimens after 3 days culturing period

IV. Conclusion

In this study, a biomimetic system was designed by using a mechanism inside gelatin hydrogel and similar temperature and pH conditions of the body. Additionally, the aim was to analyze the effect of Zn ion on its structure and properties. Because of the used mechanism, the calcium phosphate phase was formed in the base phase, and it created a chemical bond with the base phase. Moreover, sediment particles were completely distributed in the base phase, and their dimensions were about 100 nm and had an almost spherical shape. It should be noted that the degree of cell viability and ALP activity in the presence of 5 mol. % Zn ions (5ZnBG/G) increased. However, with the increase in the amount of Zn up to 10 mol. % in the composite scaffold, cytotoxicity effect, and ALP activity have increased and decreased, respectively. Therefore, the results indicated better bone cell activity and bone conduction of 5ZnBG/G compared to other specimens. In fact, it confirmed the high capacity of this scaffold for use in bone tissue engineering (BTE).

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