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ORIGINAL PAPER

Synthesizing and Characterizing of Gelatin-Chitosan-Bioactive Glass (58s) Scaffolds for Bone Tissue Engineering

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Abstract Nowadays repairing and regenerating of lost or damaged tissue still remain an important challenge in clinical techniques. Due to the variety of available bone grafts, different types of biodegradable materials are being utilized as a scaffold implant. The basic structure of the bone is an excellent natural composite which contains varieties of polymers and ceramics; therefore, it is important to manufacture a bone scaffold featuring sufficient mechanical strength, a good degree of biocompatibility, biodegradation and an increased rate of formation of new tissue. Bioactive glass has an appealing characteristic which can be utilized for repairing purposes as well as to cause a rapid response from the bone graft. In this study, a composite scaffold based on polymer matrix (gelatin-chitosan) and bioactive glass 58s was synthesized in the laboratory. Five samples of polymer scaffold with different proportions of bioactive glass were designed and investigated. The scaffolds were dried with freeze dryer, and a spongy structure was generated. The composite survey was carried out through FTIR technique to examine the crystallization of the structure, XRD to examine the morphology of the porosities, and SEM to examine the size of porosities and formation of apatite. This study reveals that the size of porosities is about 170–320 μm, which is suitable for angiogenesis and cell growth in the bone. The combination of enhanced properties and the formation of apatite on the surface of the scaffolds make them an ideal option as a bone substitute.

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Keywords Tissue engineering · Bone scaffold · Chitosan · Gelatin · Bioactive glass

1 Introduction

Organ transplantation is still a major problem in different types of surgery, and grafts are being widely used in various fields encompassing—but not limited to -orthopedic, neurosurgical and periodontal surgery [1, 2]. This issue highly motivates researchers to perform extensive studies on the subject. Not only bone graft utilization, but also the application of appropriate biocompatible materials would be useful in treatment of bone injuries. Thus, by combining tissue engineering and biomaterials, osteoblast cells are being tried to restore normal biological activities in bones [3].

Various methods have been proposed in bone tissue engineering such as autograft, allograft, and xenograft. Each biological graft has certain deficiencies including a lack of resources, need for further surgery and augment the risk of morbidity, invasive collecting method of host tissue, high risk of rejection, immune system stimulation, sepsis and pain, impairment of the nervous system, and last but not least, the high cost of surgeries [4–6].

However, the statistics indicate that over two million instances of bone grafting are applied as a cure for bone defects around the world each year. Due to significant problems in orthopedic surgeries, synthetic bone scaffolds are usually regarded as better and more efficient in terms of bone regeneration [7].

The first step towards understanding bone tissue engineering is apprehending the structure and function of tissue formation on different levels. Bone is the only organ able to regenerate and restrict itself in small and large scales.

This process is carried out by creating a delicate balance between the processes of bone formation (Osteogenesis) and bone loss (Osteoclasis) [8]. The bone can adapt itself to new mechanical environments by changing the balance between osteogenesis and osteoclasis. These processes react to the dynamic stress, and as a result, if it is greater than the physiological stress, the bone formation rate will increase. Conversely, if a lower dynamic stress than the physiological one is enforced, the balance between the two osteogenic and osteoclastic processes will be lost and geared towards osteoclasis [9].

Bone tissue engineering is based on the simulation of bone healing and bone formation processes in the laboratory. Though bones have a three-dimensional network, their cells cannot grow and reproduce in laboratory environments in all three dimensions by themselves. Thus, a three-dimensional structure (a structure similar to bone scaffold) is required to grow bone cells on the bone's surface [10].

The main elements in bone tissue engineering are cells, osteogenesis, and cellular matrix. Mineral tissues such as bone and teeth naturally consist of composite structures that have mechanical, chemical and biological properties. In order to restore a hard tissue, a three-dimensional composite is needed as a scaffold with an interconnected network and high percentage of porosity. The pores allow the cells to repair the tissue as they facilitate the transportation of required nutrients and angiogenesis in these areas. Therefore, bone defects can be reconstructed without losing any chemical properties using a bone tissue, and without any need for a permanent implant [11, 12].

An ideal scaffold for bone tissue engineering includes a proper rate of degradation and pore size to increase the possibility of providing cell growth within the scaffold. Bone tissue engineered scaffolds must also have good mechanical properties with repeatable forming processes. Moreover, the scaffold should be fabricated in such a way as to let bones grow in and around it, while also be capable of carrying growth factors and cells in the scaffold [13].

Research has shown that ceramics are difficult to stand alone as biomaterials due to shortcomings such as high brittleness, high sensitivity, high melting point, etc.: drawbacks that make ceramics unfeasible for application as bone tissue scaffold [14]. However, when combined with certain polymers and composites, ceramics can be rectified to have better mechanical properties, better degree of biocompatibility, the possibility to control the degradation, and increase the ability of Osteogenesis [15–17].

The bioactive glass was discovered by Hench et al. in 1969 [18]. Bioactive glasses have some benefits over other bio-ceramics; for instance, the ability to make a chemical bond with the surface layer of the scaffold and natural tissues. Moreover, apatite will form much faster on scaffolds that have bioactive glass in their structure. Finally, bioactive glass can enhance adhesion to hard and soft tissue [19, 20]. Nevertheless, phosphate-based glasses have poor flexibility and fatigue strength, so they are not suitable options when it comes to bearing the weight of a bone [21].

The other group of materials which have been studied for tissue engineering applications are polymers. Cross-linkable polymers can form a 3D Network and also have the ability to play a role as the cellular matrix. This environment is a suitable place for transportation of nutrients and by-products of cell metabolism [14].

Polymers have a number of specifications including biocompatibility and in situ polymerization, but there is also evidence against their use as a bone scaffold, such as their lack of mechanical strength [22–25].

As described above, both polymers and ceramics have some deficiencies in terms of the required characteristics of a bone scaffold. To address this problem, composites have been developed that combine the desirable specifications of both groups of materials simultaneously to achieve an ultimately synergistic effect. As a result, this study focuses on the characterization of a new composite featuring gelatin-chitosan as polymers and bioactive glass 58s for bone tissue engineering.

2 Materials and Methods

In this section, the materials used for strengthening the composite material as well as the procedure are discussed.

2.1 Materials

Due to the numerous advantageous features of Gelatin and Chitosan, they are widely used in bone substitution. However, when used alone, they lack good mechanical properties and ability to act as a bone replacement. Consequently in order to tackle with these drawbacks, in this study, the bioactive glass is investigated as an underpinning phase [26].

Microbiological Chitosan (low molecular weight) was purchased from Sigma Company (USA). Gelatin for Microbiology 1.04070.0500 (Cat No. 9000-70-B) was supplied by the Merck Company (GERMANY). Bioactive glass 58 S has been synthesized in the AUT laboratory. In addition, acetic acid (Merck, Cat No. 100062), and distilled water were used in this experiment.

2.2 Preparation of Composite Scaffold

Five samples of scaffold with different proportions of bioactive glass have been provided as below. To prepare a 5% solution of gelatin, weighed gelatin was added slowly to distilled water at 40 °C. After thorough dissolution of the

gelatin, it is put aside until its temperature was decreased to normal.

Chitosan was dissolved in 1% acetic acid solution. This solution was added to the prepared gelatin and mixed using a magnetic stirrer for 40 min at 40 °C and 100 rpm. Bioactive glass powder was added to the mixture and stirred until it seemed to be completely dispersed. Then, the mixture was poured into the petri dish and was refrigerated for 24 h. Later, it was placed in the freezer for 24 h.

The freeze-drying method has been used in this study. In the first phase, the polymer solution was frozen at a low temperature until all material become ice crystals. In the second stage to perform complete drying, samples were in a freeze dryer for 24 h at temperature of − 55°C. Freeze drier evacuates the inside air and produces a vacuum within some minutes, as a result, and with the help of a vacuum pump, pressure has been enforced. Consequently, the solvent is sublimated. Sublimation of ice crystals establishes a very porous sponge structure in the scaffold.

Five Samples were prepared by undergoing the above procedure. For characterization of samples (XRD and FTIR tests), it is required to make a very soft powder from each scaffold. So some necessary amount of each sample was crushed into powder by a soft rasp.

Moreover, to study the samples functions in a similar biological situation as the human body, pieces with the same dimensions for all samples were prepared and were held in SBF (simulator body fluid) for 7 and 14 days in incubator (in an environment similar to the biological human body and at 37 °C temperature). The samples were excluded from the SBF solution and were dried by the freeze dryer. Then samples were powdered for after SBF studies. The instruction similar to T. Kokubo, H. Takadama was used to prepare the SBF solution [27].

2.3 Physical and Morphological Properties

2.3.1 Fourier Transform Infrared

In order to determine chemical bond and composition, FTIR spectrometer (NEXUS) 670 model was used [28]. 1 mg of each sample were mixed completely with 300 mg potassium bromide (KBr) and then pelleted. It was later investigated in the wave range 400–4000 Cm^{-1} operating in the absorption mode. FTIR was studied for all the five prepared powder samples both before and after immersing in SBF solution.

2.3.2 X-ray Diffraction

Furthermore, for crystal characterization of the prepared samples, X-Ray diffraction (XRD) was used. XRD is an old technique and is used to characterize crystals [29]. It determines the crystal structures such as the geometry of network, unknown materials, crystalline phases and size measurement. The crystal structure can be formed to investigate the physiochemical properties. XRD of samples was obtained at room temperature using Intel Equinox 3000 operating at 40 kV voltage and the angle range of 5–118°.

2.3.3 Scanning Electron Microscope

The morphology size and shape of the pores were examined with Scanning Electron Microscope (SEM). The samples were coated with a thin layer of gold with cathode sputtering method using Seron technology AIS2100 with 0.5 kV ∼ 30 kV.

2.4 Mechanical Property Assessment

The mechanical properties of the samples were analyzed using compression tests dynamic testing machine (DTM) HCT 400/25. The pieces with the determined dimensions were loaded at a rate of 0.05 mm/sec ∼3 mm/min until failure. The Young's modulus was calculated from the slope of the linear region of the stress–strain curves in the 0–1% strain range. This process was applied to all five samples.

2.5 Measuring Water Uptake

Water absorption and inflation increase the pore size, which makes it easy for oxygen and nutrients to transport to the interior sides of the scaffold. But if inflation is performed uncontrollably, it will not be applicable in tissue engineering because it causes the cell growth to stop. However, the inflation and water absorption can be controlled by increasing the glass amount in the scaffold.

Here the dry weight of the scaffold was noted by w_0. Scaffolds were placed in 10 ml water for 2 h and then removed and placed on the filter paper and wet weight was recorded as w_1. The ratio of water uptake was calculated for five samples using the Eq. 1

$$Wu = \frac{w_1 - w_0}{w_0} \times 100 \tag{1}$$

2.6 Statistical Analysis

Constantly investigations were led clinched alongside fifth replication. Those results were provided for concerning illustration methods Standard Error (SE). Measurable dissection might have been conveyed out toward applying one-way ANOVA and Tukey test .The point is considered when $P < 0.05$. Besides, for contemplating of gathering normalizing, Kolmogorov–Smirnov test have been effectively utilized [30].

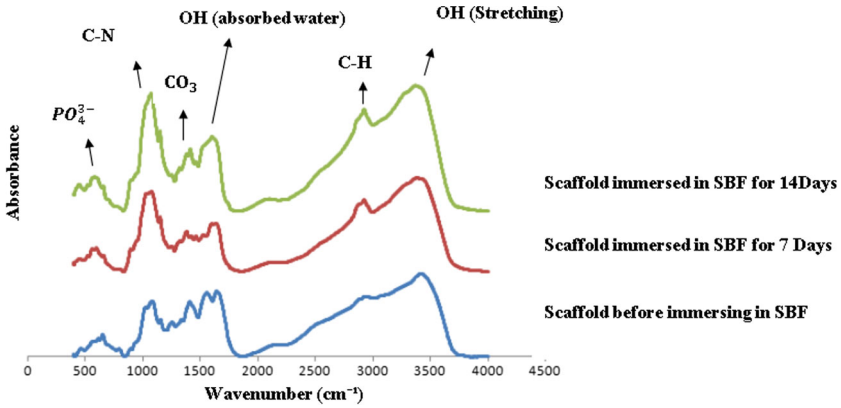

Fig. 1 FTIR Spectra of the prepared scaffold before and after immersion in SBF solution

3 Results and Discussion

In this section, the results of the tests explained above are explored. For this purpose, we study the FTIR and XRD test results consequently, and then the mechanical properties of the samples are investigated.

3.1 FTIR Analysis

The FTIR investigations were carried out in absorbance mode. It can be seen obviously that the range of absorbed waves are various well under 500 cm^{-1} to 4000 cm^{-1}. The absorbed wave numbers can be categorized into three groups: the wave numbers of polymer matrix (gelatin and chitosan), the wave numbers related to ceramic phase (bioactive glass 58 S) and the wave number related to these two phases bonding [31]. Figure 1, illustrates the results of the FTIR test for each sample before immersing in SBF solution and after being immersed for 7 and 14 days in SBF solution. The band at 460 shows the presence of PO4 and the wave number of 1070 related to Si-O-Si which is the group of bio glass. The figures represent that two peaks take place at 3450 cm^{-1} and 650 cm^{-1} that are attributed to O-H. This indicates the presence of hydroxyapatite in the structure. As the amount of ceramic phase increases, the O-H peak climbs to a higher value. The wave number at 1563 cm^{-1} and 1600 cm^{-1} correspond to the functional group in the polymer matrix (gelatin and chitosan) which indicate double bond C=O and N=H. FTIR spectrum for composite samples shows a wave number at 2800 cm^{-1} due to the chemical bond of PO and OH, while a decrease in the non-composite sample can be observed as a result of the involvement of OH and matrix phase. Furthermore, the spectrum at 1407 cm^{-1} represents the constitution of hydrogen bonds between COOH of polymer phase and OH of ceramic phase. The COOH group of gelatin in the scaffold may exist in the form of COO. In other words it corresponds to the bond between carboxylate in gelatin and Ca^2 in bio glass. The peak waves at 1650, 1220 and 1550 are attributed to the amid I, amid II, amid III respectively. The constitution of hydrogen bonds between NH of polymer phase and OH of ceramic phase leads to a heterogeneous aggregation of HA particles, which results in heterogeneous evaporation of the solvent. Hence, smaller pores are observed in the places

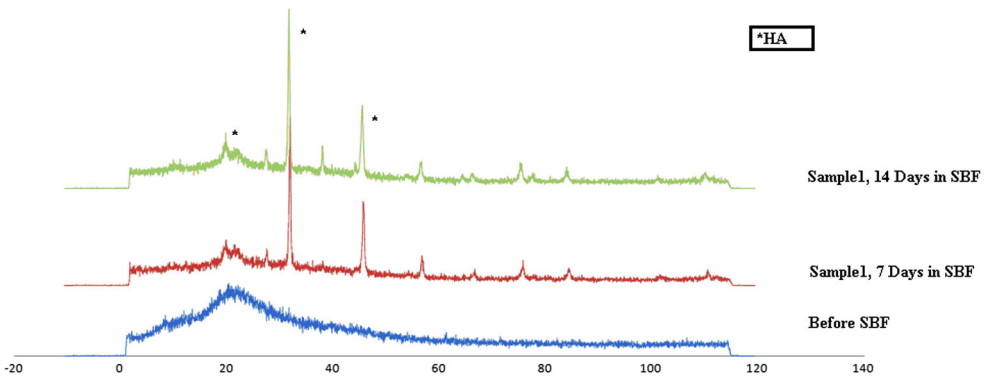

Fig. 2 The XRD spectrum for Sample 1

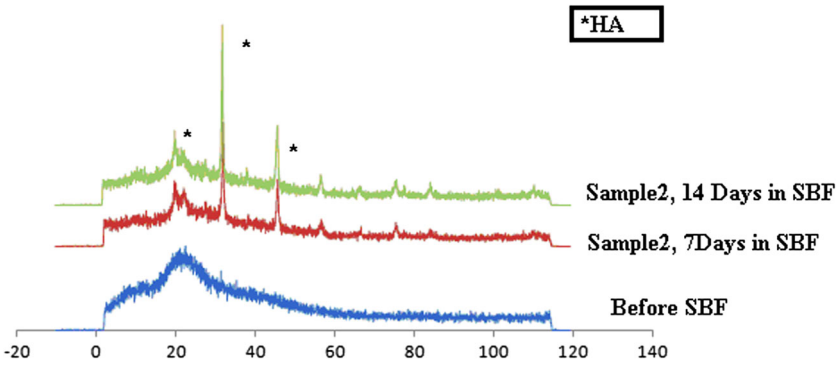

Fig. 3 The XRD spectrum for Sample 2

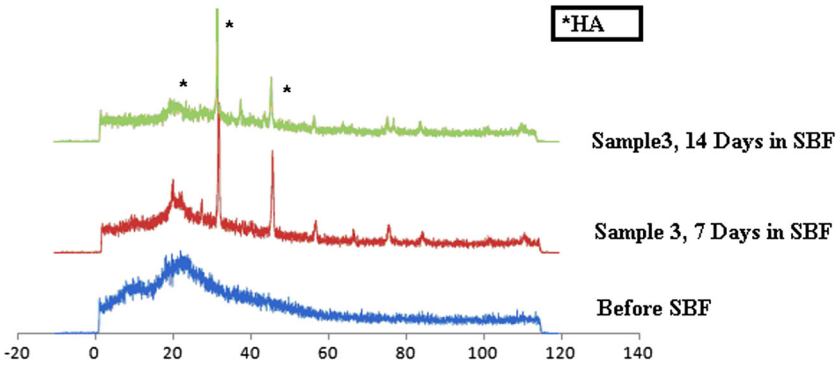

Fig. 4 The XRD spectrum for Sample 3

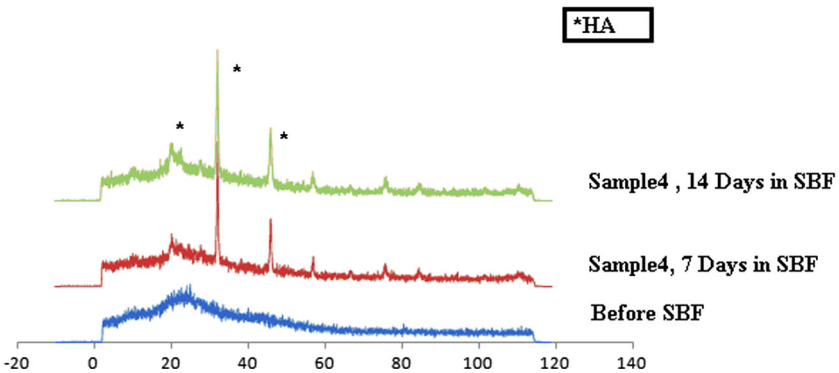

Fig. 5 The XRD spectrum for Sample 4

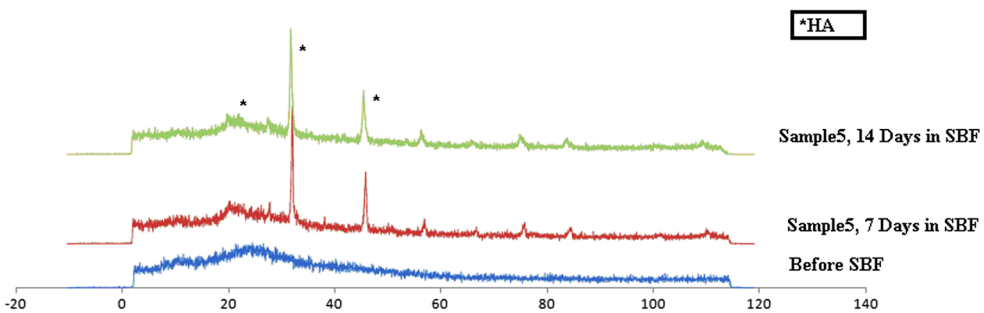

Fig. 6 The XRD spectrum for Sample 5

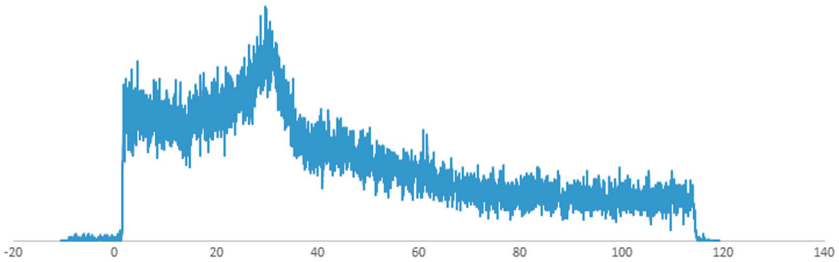

Fig. 7 The XRD spectrum for bioactive glass 58 S

with lower evaporation. This heterogeneity of the pores is seen only in the cross-sectional SEM which is probably dependent on the direction of solvent exhaustion.

3.2 XRD Analysis

As can be seen clearly, the XRD was studied in 2θ and in a range from 0 to 120° in the room temperature. The XRD spectra of composite scaffold show no diffraction peak the broadness of the band indicate the amorphous nature of the bioactive glass (Fig. 7). The XRD pattern relates to the scaffold before soaking in SBF solution. It confirms that the prepared scaffold exists in amorphous state and no crystalline peak is observable and the matrix phase has a slight peak that is not visible. Contrasting Figs. 2, 3, 4, 5 and 6 show the results of the XRD test for each sample before and after immersion in SBF solution for 7 and 14 days, a peak is observed in $2\theta = 31°$ which means that HA has been formed sufficiently on the surface of the scaffolds (JCPDS#09-432) [32]. After immersing in SBF solution, the SBF penetrates through the scaffold structure and attacks the amorphous part, so long chains break down, but crystallite preserves the structure of the scaffold. Due to the combination of the ceramic phase with matrix phase, the peaks have low intensity, and some noises are visible. No peak is present in XRD charts related to the samples, which has not been in the SBF solution. XRD of the scaffold shows a broad peak between 20° and 40° that represents the existence of matrix network in the scaffold. In general, chitosan structure has hydroxyl and amine groups, which can make strong hydrogen bonds. Consequently, chitosan has a high crystalline structure when chitosan and gelatin make a network, crystalline decreases due to the changes in hydrogen bonds which causes a wide peak in XRD. Moreover, increasing the glass in scaffolds made less intensive peak. This reveals that the existence of glass makes the intermolecular interaction intensely weak.

By increasing, the number of days that scaffolds have been immersed in SBF solution, the peak intensity of hydroxyapatite becomes sharper, which is caused by the formation of hydroxyapatite on the surface of the scaffold, and it is a sign of good bioactivity. This can be seen by the number of days of exposure to simulated body solution in figures of XRD studies (Fig. 7).

Table 1 The Comparison of Young modules in the scaffold

Young modules in samples		
Samples no	Ceramic phase presentation	Young modules (Mpa)
1	0	2.5 ± 0.1
2	10	3.2 ± 0.1
3	20	5.3 ± 0.3
4	30	9.7 ± 0.4
5	40	11.6 ± 0.5

3.3 Mechanical Properties Studies

An ideal scaffold should have sufficient mechanical strength outside the body to withstand the physiological environment so that it also exhibits favorable strength when placed in

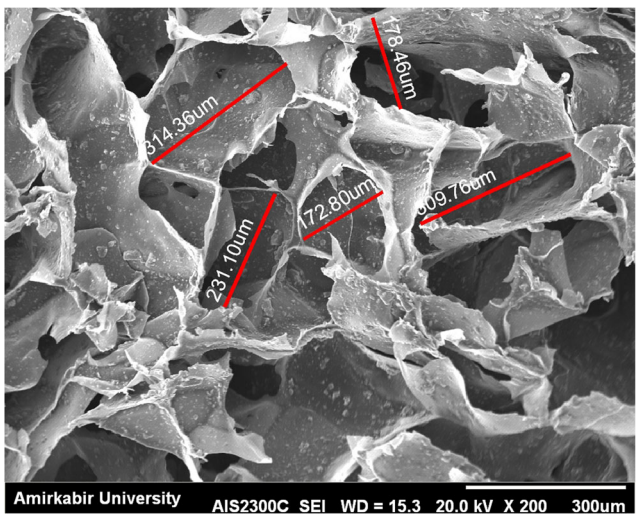

Fig. 8 The SEM Imageshoed the size of pore

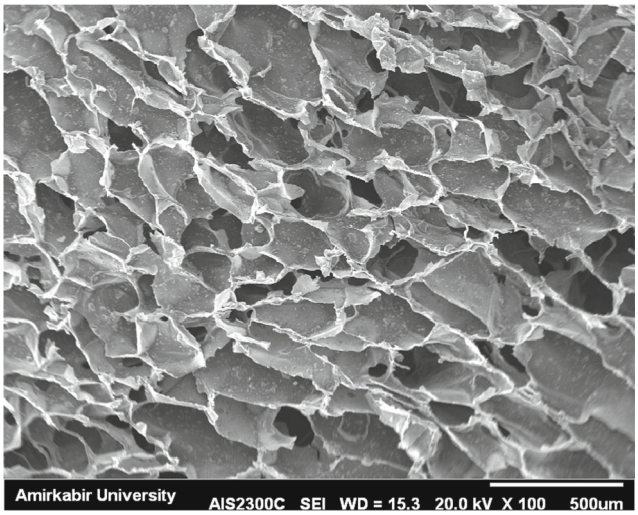

Fig. 9 SEM image with 100 × magnification before being in SBF solution

tissues, especially the ones that are load bearing. The high ratio of surface to volume is useful since it provides more possibility for connecting cells to each other. It will also allow more cells to migrate within the scaffold structure. Although the pore diameter and cross-sectional area has linearity, it requires supplying necessary mechanical stability for the scaffold, which is dependent on the scaffolds application. Furthermore, the mechanical properties are usually described by Young's modules which measure the amount of deformation under load, which is a significant factor in bone tissue engineering. It is apparent that the matrix polymer is too soft to withstand even low load and critically large deformation takes place. This fact indicates low Young's module. On the other hand, the materials such as bioactive glasses or hydroxy appetites are too brittle and deform at the particular load.

Strain and stress values have been calculated at any moment for each curve. It can be observed that Young's modulus grows with increasing the ceramic phase. Mechanical properties are significant in tissue engineering applications. This is because, for instance, if mechanical strength for tested scaffold is very low then it is not suitable for applications under load. This low strength may be the result of high porosity in the scaffold, different portion of the ceramic phase, lack of cross-linked would be the reasons. Soaking the scaffolds in glutaraldehyde solution (1%) would be a way to enhance mechanical behavior.

The comparison between primary and secondary weight is given in Table 1. It shows that by increasing the amount of glass, water absorption has decreased because the ceramic phase of the scaffold has been increased.

3.4 SEM Observation

The presence of the ceramic phase in the polymer-ceramic composite decreases the porosity of the scaffold. The initial freezing temperature and lack of uniformity of parameters in all compartments of freeze-drying are among the influential factors of this fact. Increasing the rate of porosity allows the cells to make use of the internal surface of the scaffold. Since the porosity size should be proportional to the culture cells, this size should under control which can be yielded

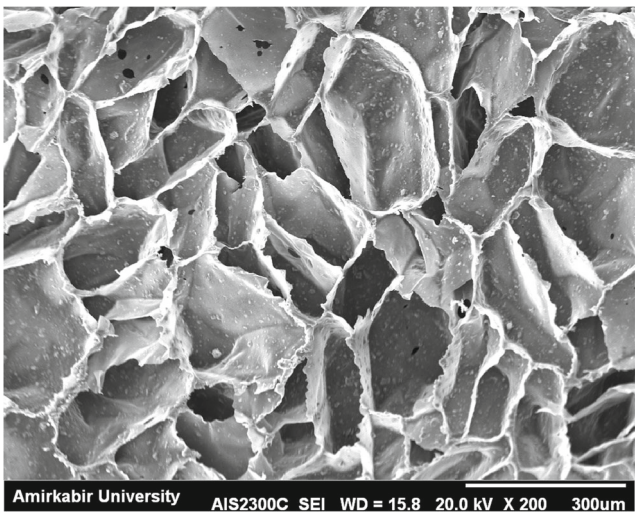

Fig. 10 SEM image with 200× magnification before being in SBF solution

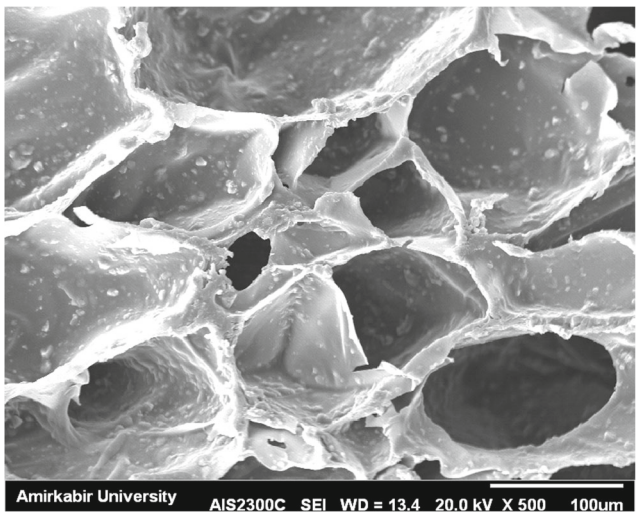

Fig. 11 SEM image with 500× magnification before being in SBF solution

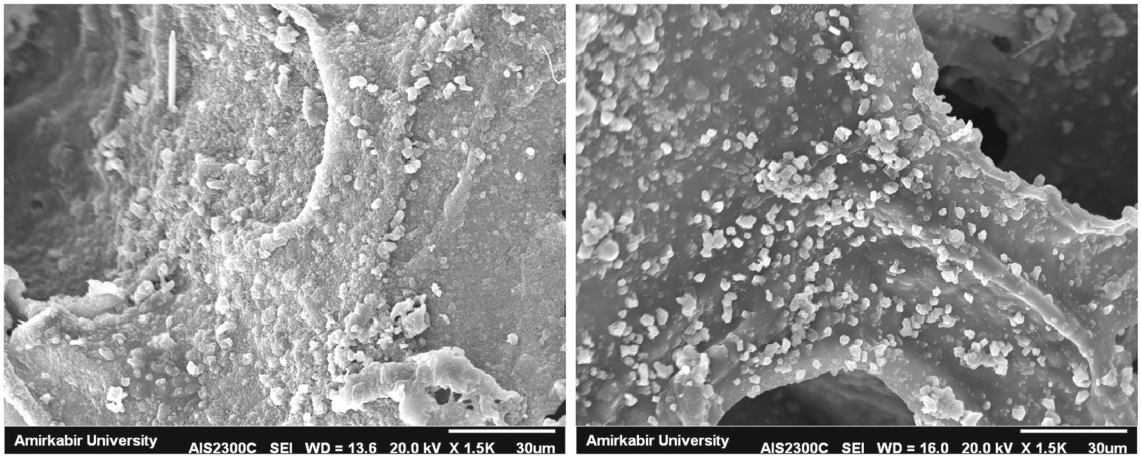

Fig. 12 The SEM images for the scaffold which has been in SBF solution for 7 days

by close supervision of items such as construction method, devices parameters, and types and amount of ingredients.

The gelatin substance generates higher viscosity. Therefore when the bioactive glass particles are added, it may result in lack of glass dispersion in the scaffold; this is due to the prevention of the movement of chains in the polymer as a side issue of high viscosity, and may lead to a heterogeneous solution. This implies that the number of close pores will be greater than the number of open pores in the solution.

According to SEM images, the pore size can be estimated, as it is illustrated in the SEM images. The pores have the form of hexagonal honeycombs in beehives and are interconnected. Bone formation is highly dependent on angiogenesis, which according to the researchers are suitable for bone tissue engineering since the hole size required to create angiogenesis between 170–320 micrometers Scaffolding with a porous structure with such dimensions is effective for this purpose (Figs. 8, 9, 10, 11, 12 and 13).

3.5 Water Uptake

Gelatin has high water uptake specification because of having a functional group in its structure such as amines, carboxyl, and hydroperoxide. Moreover, by increasing the ceramic phase, the water uptake decreases. Increasing the water uptake also allows the cells to reach more nutrients, but it results in a reduction in the mechanical properties. Consequently, the rate of water absorption should be controllable in an ideal bone scaffold. The water uptake studies was performed in PBS (pH 7.4) at 37 °C. The amount of water uptake in the scaffold has been shown in Table 2.

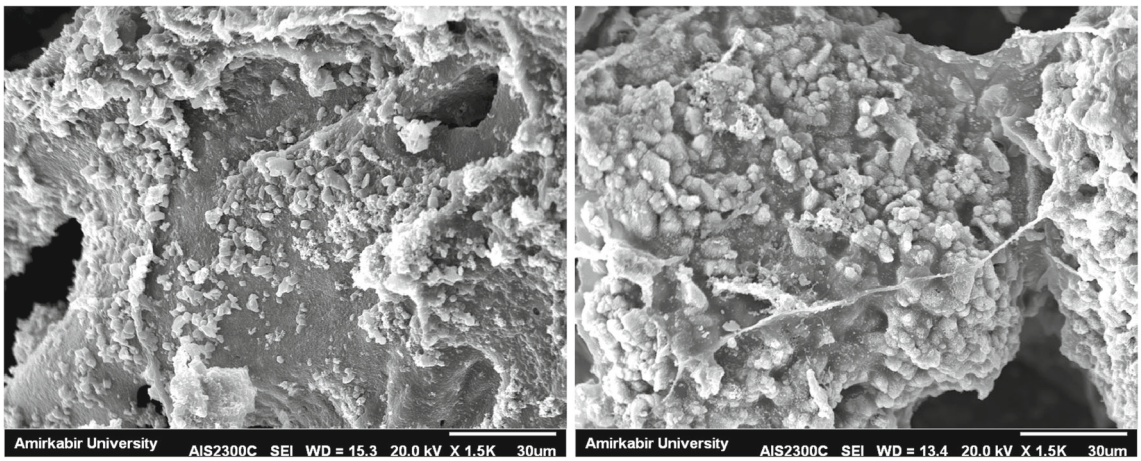

Fig. 13 The SEM images for the scaffold which has been in SBF solution for 14 days

Table 2 The amount of water uptake in the scaffold

Water uptake rate			
Sample no	w_0	w_1	wu
1	0.0571 ± 0.0028	0.252 ± 0.012	341.33 ± 3.41
2	0.0544 ± 0.0032	0.209 ± 0.010	284.19 ± 2.84
3	0.0549 ± 0.0038	0.155 ± 0.011	182.33 ± 3.64
4	0.0549 ± 0.0027	0.132 ± 0.007	140.44 ± 1.40
5	0.0547 ± 0.0021	0.116 ± 0.004	112.07 ± 2.24

4 Conclusion

In this study, a 3D scaffold has been prepared by gelatin-chitosan as the matrix polymer and bioactive 58S glass as the ceramic phase by use of the freeze-drying method. Good chemical bonding was created between bioactive glass and polymer phase, and after freeze-drying, regular and interconnected pores has been developed with proper size that results in angiogenesis and later, cell growth.

Some tests have been performed to study the morphology by system that leads to measuring water uptakes, the structure and bonding by FTIR and XRD. The peaks in the results of these test indicate the creation of HA in the scaffolds. The SEM photos suggest the size and connection of the pores. These scaffolds can be used as a replacement of bone in bone tissue engineering since they have proper pore size and their similarity to the bone structure.

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