Study on Compressive and Compatibility Properties of Titanium Functionally Graded Scaffolds as Bone Replacements

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ABSTRACT

Recently, Functionally Graded Scaffolds (FGSs) have attracted a lot of attention as bone replacements due to their gradient porosities as well as a bone structure. In present study titanium functionally graded scaffolds (FGSs) were fabricated by powder metallurgy route using Mg and carbamide as space holders. The arranged layers with 20, 40 and 60 Vol.% porosities were compacted in steel die using uniaxial pressure of 500 MPa before sintering in sealed quartz tubes at 1100 °C for 3 hours. Image analyzing results and scanning electron microscope (SEM) observations showed more regular shapes and sizes of pores in FGSs using Mg as a space holder compared to carbamide. The observed compressive strength and Young's moduli of the FGSs having Mg as a space holder were in the range of 47- 160 MPa and 25-75 GPa , respectively which can be appropriate as bone replacements. The results of MTT assay showed that the values of proliferation rate were higher in samples produced using Mg.

1-Introduction

Poor mechanical properties of ceramic and polymeric biomaterials led researchers to focus on metallic biomaterials for load bearing application[1]. Nowadays titanium (Ti) and its alloys such as Ti-6Al-4V have been widely used due to their good mechanical properties, wonderful biocompatibility and high corrosion resistance [2-10]. Higher Young's moduli of metals compared to bones is the main defect of these materials as bone replacements which leads to stress shielding[11-19]. Employing porous materials has been known as a solution to solve this problem in medical application[12]. The bone has heterogeneous structure with a gradient porosity. Therefore functionally graded materials (FGMs) with heterogeneous structures could be good candidate for bone replacements[20]. These structures must possess high porosity in the inner parts similar to cancellous bone and low porosity in the outer parts similar to cortical bone as well as a bone structure[21].

Among various methods to manufacture FGMs, powder metallurgy (PM) route with space holder technique is widely used due to its considerable advantages such as low cost and better control. In this method, pores' characteristics are function of powder size and shape as well as the shape and size of space holder[6]. The gradient porosity could be obtained via layers arranging technique[22]. Some of the space holder materials contain carbamide (urea), sodium chloride, ammonium hydrogen carbonate, and

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magnesium [1, 6, 23, 24]. Although carmabide has been used in a lot of works, magnesium could provide a reducing atmosphere, which prevents the titanium from oxidation during the sintering process[25]. Furthermore its solubility in titanium is expected to be negligible[26, 27]. Thus magnesium is suitable in titanium scaffolds production. Powder metallurgy route (by layers arranging) as one of simplest way to produce functionally graded scaffolds among different complex methods was employed in this study. Moreover, comprehensive comparison between carbamide and Mg as space holders and resulted mechanical and biocompatibility properties are described in this article.

2-Materials and methods

2-1-Raw materials

Ti-6Al-4V, carbamide, and magnesium (Merck Germany) were sieved separately to obtain powder range of 45-90 μm for the alloy and 90-180 μm for carbamide and Mg powders.

2-2-Fabrication of FGSs

The first stage of producing FGSs by PM method is layers arranging. The layers arranging were performed in three layers: the first, second, and third contain 20, 40, and 60 Vol.% of the space holder, respectively. Each layer was mixed with the alloy powder in an agate mortar using 5 Wt% Poly Vinyl Alcohol (PVA) solution (5Wt% PVA + 95Wt% water) as the binder prior to compaction. In order to reach homogenous porosity distribution in each layer, PVA solution was added to mixture [25]. The arranged layers were uniaxially pressed at a pressure of 500 MPa in a steel die using a hydraulic press. The heat treatment process consisted steps, i.e. at 450 °C for 2 hr to allow debinding of PVA and evaporating of carbamide and then 1100 °C for 3 hr for evaporating of Mg and sintering process, simultaneously. The main phases in the porous sample with Mg as a space holder were characterized by X-Ray Diffraction (XRD Philips X'PERT MPD) analysis using a Cu-Kαradiation source. Production process involved in the space holder technique applied is summarized in Fig .1.

2-3-Pore characterization

The amount of porosity in FGSs was determined by image analyzer attached to an optical microscope and suitable analyzing software (Image Tool). Pore characterizations such as pores shape and size were analyzed on the sectioned parts of the prototypes using this software. Furthermore, FGSs were also observed using scanning electron microscope (SEM).

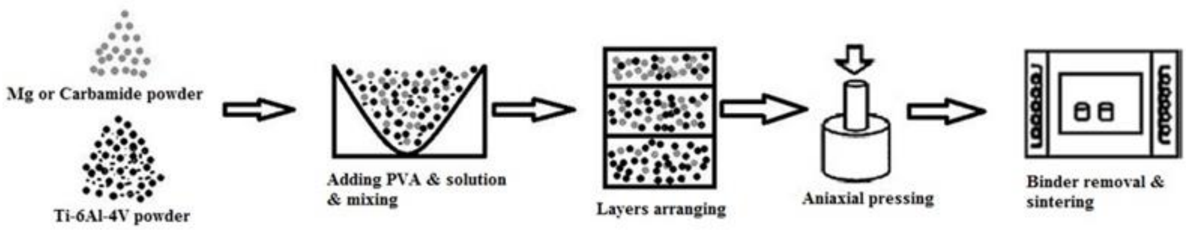

Fig. 1. Schematic of processing steps for fabricating FGSs.

2-4-Mechanical testing

Compression tests were carried out on samples contain 20, 40, and 60 Vol.% of space holders separately with diameter and height of 10 mm at 25 °C at the strain rate of 0.5 mm/min according to ASTM 2150-13 standard employing Instorn 1185 universal test machine. To compensate the possible scattering of the results three specimens were tested at each porosity level. The Young's moduli of the samples were calculated from the stress-strain curves fitted to the linear elastic regions. The average compressive strength and elastic moduli for each sample were reported.

2-5-Cell culture

Prior to cell culture, FGSs were cleaned ultrasonically in acetone, 100% ethanol and deionized water for 15 min. Subsequently they were dried with and autoclaved at 121 °C under a pressure of 1.5 Kg/cm^2 for 1 hour before placing them in multiwell culture plates.

MG-63 human osteosarcoma cell lines (NCBI C-555, National Cell Bank of Iran, Pasteur

Institute of Iran) were cultured at a density of 1×10^4 cells on each sample. MG-63 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Scotland) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100µg/mL streptomycin (Sigma, U.S.A.). MG-63 cells were harvested with 0.25% trypsin-EDTA solution (Sigma) in phosphate buffered saline (PBS, pH 7.4). According to the ISO 10993-5, MG-63 cells were incubated at 37 °C in humidified air with 5% CO_2. The cultured medium was changed every two days.

2-5-1-Cell morphology

MG-63 cells at a density of 5×10^3 cells/well in 24-well plates were placed on each FGS. The plates were incubated at 37°C in humidified air containing 5% CO_2. 2.5% glutaraldehyde buffered in 0.2 M phosphate-buffered saline (PBS) was used for fixing cells after 6 h of incubation period. Graded alcohols (50, 70, 90, 95, and 100%) were used for dehydrating specimens. Dehydration was carried out by air drying for 24 h. Morphology of the osteoblast cells which were attached to the samples were observed by means of SEM after sputtering a gold layer.

2-5-2-Cell proliferation assay

Dimethylthiazol diphenyl tetrazolium bromide (MTT) assay was used to determine the proliferation rate of osteoblast cells. Briefly, MG-63 cultured cells were suspended in culture medium at a density of 1×10^3 cells/50 µL and were added to each of the wells in the 96-microtiter plates (NUNC, Denmark). The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 h. The FGSs with different amounts of magnesium and carbamide were placed on the MG-63 cells. One tissue culture polystyrene (TPS) well used as a control. The plates containing FGSs and the cells were incubated at 37°C with 5% CO_2 for 7 days. The cultured medium was changed every three days. At predetermined intervals, FGSs were taken out of wells. 100 microliters of MTT (5 mg/mL in medium) was added to each of the wells and incubated for another 4 h at 37°C in a humidified atmosphere of 5% of CO_2 in air. At this stage the MTT was removed and the formazan crystals were dissolved by adding 100 µL of isopropanol (Sigma) in each well. The plates were placed in the incubator for 10 min and then in a cold room for 15 min prior to absorbance measurements. Multiwell microplate reader (ICN, Switzerland) was used for reading optical density (OD) of each well at 545 nm wavelengths. The assay was repeated three times.

2-5-3-Statistical analysis

Statistical analysis was performed using SPSS. Data are reported as mean ± standard deviation and value were considered significant at $p<0.05$.

3-Results and discussion:

3-1-Characterization of porosity and pores

After sintering, diffraction patterns of the sample were characterized by XRD analysis and peaks were mainly Ti-6Al-4V, as shown in Fig.2. XRD patterns reveal that there was no reaction between the two original components, i.e., magnesium and titanium, during the sintering process. Typical micrograph of the fabricated FGS using carbamide as a space holder is presented in Fig.3. As can be seen, the acquired structure has stretched elliptical pores, considering that the final pores shapes depend on the primary shapes of the space holder particles. Due to agglomeration of carbamide particles before and during mixing with Ti-6Al-4V particles, pores shapes are irregular and have different sizes. The pores in the first layer with 20 Vol.% carbamide are mostly closed type while the other layers have open type pores generated after evaporation of carbamide.

Different segments of the prototype were analyzed by the software (Image Tool) after sectioning. The results of image analysis show that the amount of porosity increases along the scaffold. As it is shown in Fig.4 in layers with lower percentage of the carbamide, porosity variation is heterogeneous. This also confirms agglomeration of carbamide during the fabrication process. Fig.5. presents micrograph of the FGS fabricated by Mg as a space holder. As can be seen, the pores are mostly spherical which may be due to negligible magnesium deformation during compression, while in scaffolds with carbamide as a space holder, the pores were elliptical.

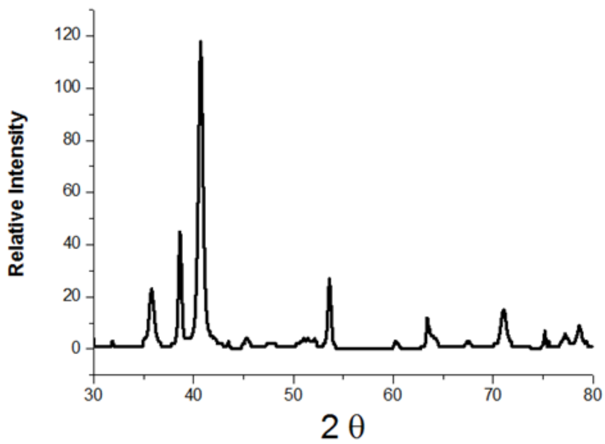

Fig. 2. XRD peaks for fabricated FGS used Mg as a spaceholder.

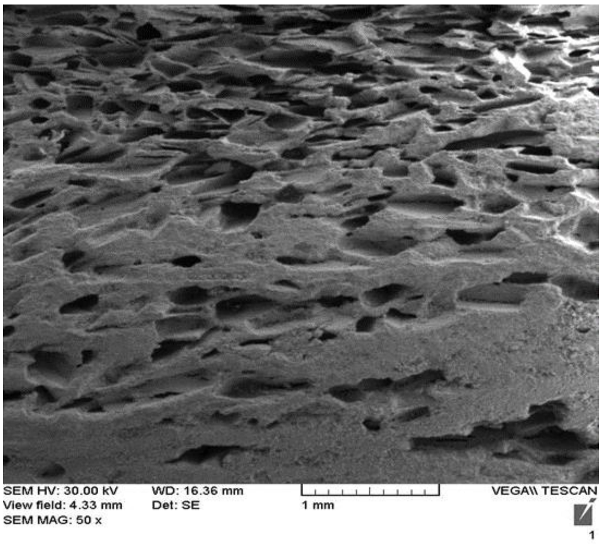

Fig. 3. SEM micrograph of fabricated FGS using urea as a space holder.

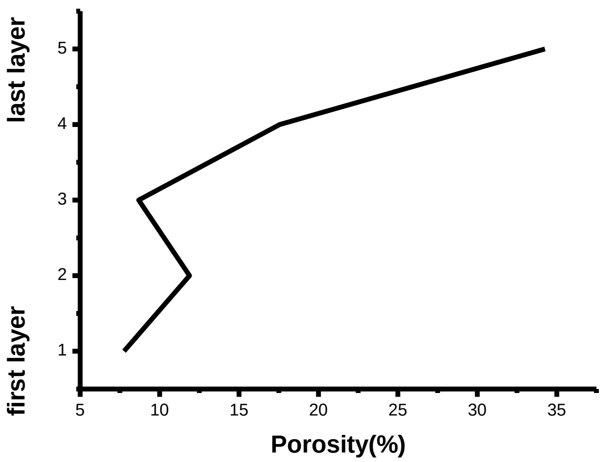

Fig. 4. variation of porosity in FGS with urea as a space holder.

Despite of using carbamide as a space holder, the pores in the scaffold fabricated with Mg as a space holder were distributed homogenously.
Also in this structure amount of porosity increased from first layer to last one, which is illustrated in Fig .6.
According to Fig.6, distributed pores were across the layers uniformly. Two types of pores were observed in the scaffolds after sintering. One was the closed type pores in the first two layers while the large pores in the last layer were interconnected which facilitate transport of nutrients and oxygen. Li et al[28] reported that higher porosity leads to increase in cell proliferation.

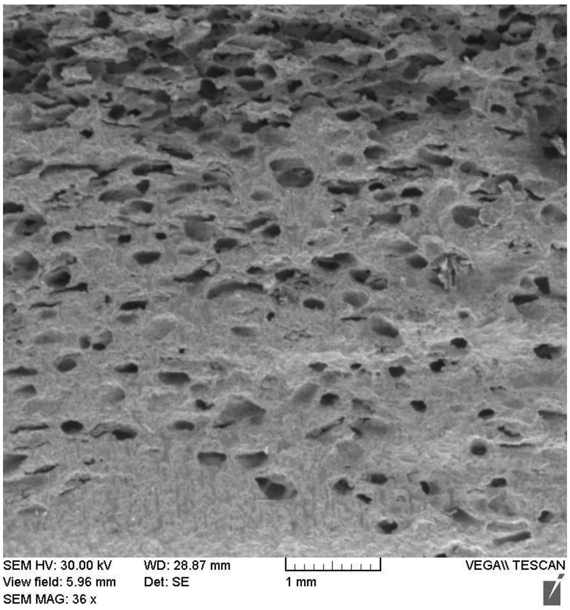

Fig 5 SEM micrograph of fabricated FGS using Mg as a space holder.

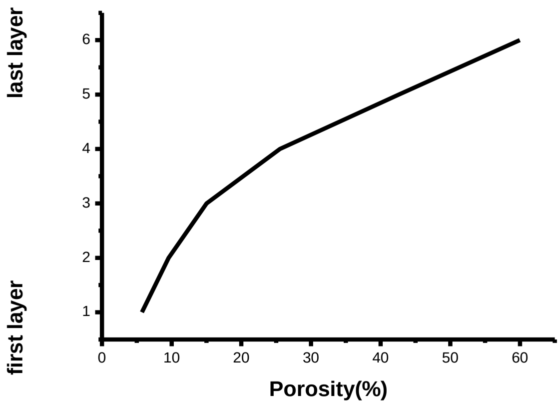

Fig. 6. variation of porosity in FGS with Mg as a space holder.

After sintering, the scaffold having Mg as a space holder was analyzed by energy dispersive spectrometry (EDS) and result is presented in Fig. 7. These spectrographs show the presence of titanium and magnesium in scaffold. It should be noted that in contrast to carbamide, the remaining Mg is not harmful for the body, since it bonds with phosphate and causes useful effects on mineralizing the bone. Magnesium stimulates the bone growth and plays useful role in ossification[29]. Furthermore, Mg can be dissolve in the body and form compounds that are non-toxic in small quantities[26]. It is believed that the minimum pore size for growth of filamentous tissues is 10-75 µm, 75-100 µm for non-mineral bone tissues, and over 100 µm for mineral ossification[30]. Therefore, it could be said that the developed scaffolds in this study enable growth of filamentous tissues, non-mineral bone tissues, and mineral bone tissues together, since they possess a wide range of pore sizes.

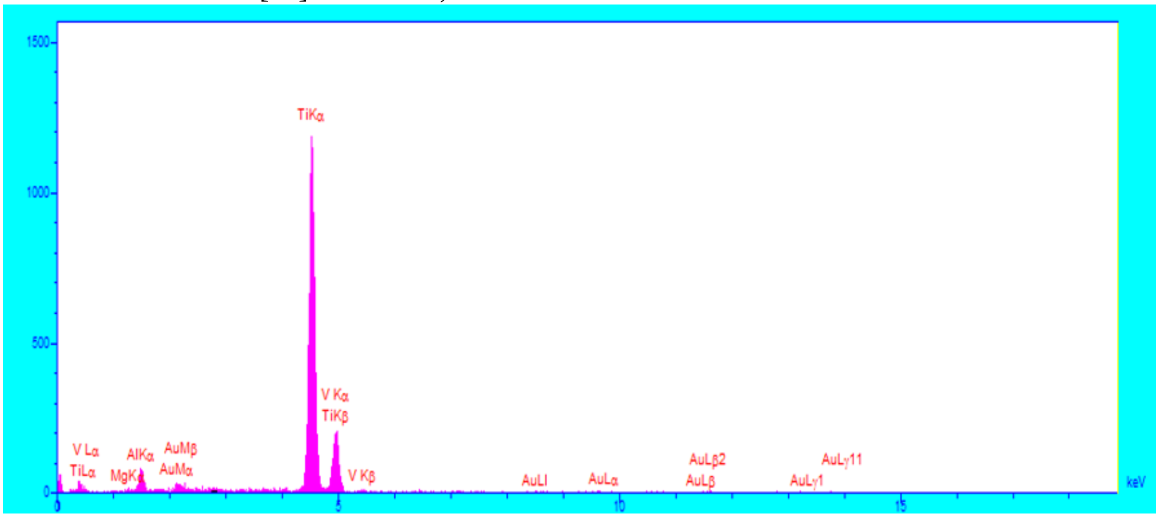

Fig. 7. EDS of fabricated FGS using magnesium as a space holder.

3-2-Mechanical behavior

Typical compressive stress-strain curves of the FGSs fabricated by different amount of carbamide and magnesium as space holder are illustrated in Fig. 8 and Fig .9, respectively. Compressive properties of the both FGSs are summarized in table 1 as a comparison. It can be seen that compressive and Young's moduli of the scaffolds are inversely correlated with the percentage of space holders. The improvement of the sintering process in scaffolds with magnesium as a space holder comparing to carbamide may be due to high chemical activity of Mg which provides a reducing atmosphere that prevents oxidation of Ti-6Al-4V during sintering according to Ellingham diagram. By comparing the values in table 1, it is obvious that using Mg as a space holder leads to proper sintering of titanium powders and better mechanical properties.

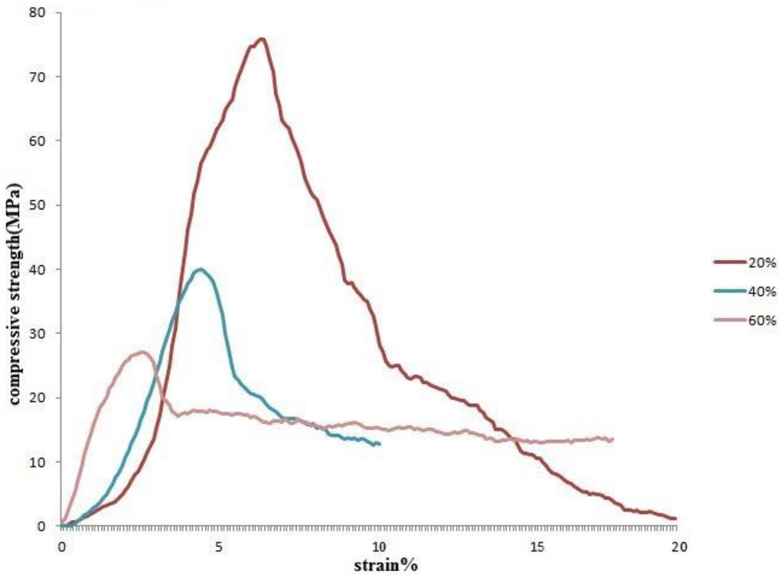

Fig. 8. Compressive stress-strain curves of manufactured FGSs with 20, 40, and 60 Vol.% porosities using urea as space holders.

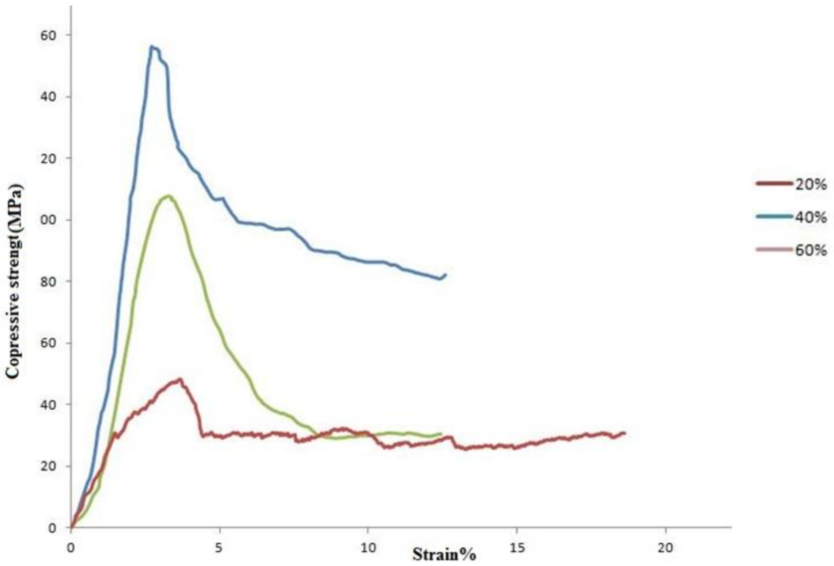

Fig. 9. Compressive stress-strain curves of manufactured FGSs with 20, 40, and 60 Vol.% porosities using Mg as space holders.

Although large total amounts of strains are exhibited in compressive stress-strain curves, the linear strains in all of the specimens are low and failing carried out in pseudo-brittle mode. This embrittlement which is the major problem of these manufactured FGSs, might be attributed to formation of brittle phase such as ω during sintering[31] and also the presence of MgO. The compressive stress-strain curves can be divided into three regions. In the first region (from the start until the yield point), the samples are compressed elastically. There is a linear relation between stress and strain in this region. In the second region (from the yield point until the maximum compressive strength), an inelastic deformation is occurred caused by shear stress along the diagonal axes to the samples at 45° to the compression direction, according to Katsuyoshi et al [32] observations. By reaching to the maximum compressive stress, the compressive strength of the samples reduces drastically, probably due to activation of non-uniformal shear bands taking place along the axes with 45° to the force applying direction. The results are in good agreement with Nishiabu [29] observations. Concentration of deformation at shear bands leads to a reduction in the load capacity of deformed samples and consequently; the third region is formed. Particle separation which starts from the corners of the cylindrical samples leads to complete failure.

It is obvious that by increasing the porosity mechanical strength and Young's moduli will be decreased. In this study, fabricated FGSs using magnesium as a space holder exhibited a wide range of compressive strength (47-160MPa) and Young's moduli (25-75) as well as bone mechanical properties that could be appropriate for bone replacements.

3-3-Biocompatibility properties

The morphology of the osteoblast cells cultured on the porous samples shown in Fig. 10 were observed by SEM. SEM micrographs show that osteoblast cells were able to attach on the surfaces of FGMs helping the formation of bridge across the pores. MG-63 cells in both samples where Mg or carbamide was used exerted good stretching ability and depicted normal shape and morphology. It could be attributed to the wide range of pore sizes presented in FGMs as well as a bone structure. The type of space holder might not have any effect on attachment of MG-63 cells. In fact, it is accepted that pore size is one of the most important factor which affects cell adhesion. It was shown that a minimum pore size of around ≈100μm is required for successful progression of the bone regeneration process [30]. Moreover Tian et al[33] have shown that MG63 cells with a diameter of 30 μm could stretch well in the pores having the size equal to themselves.

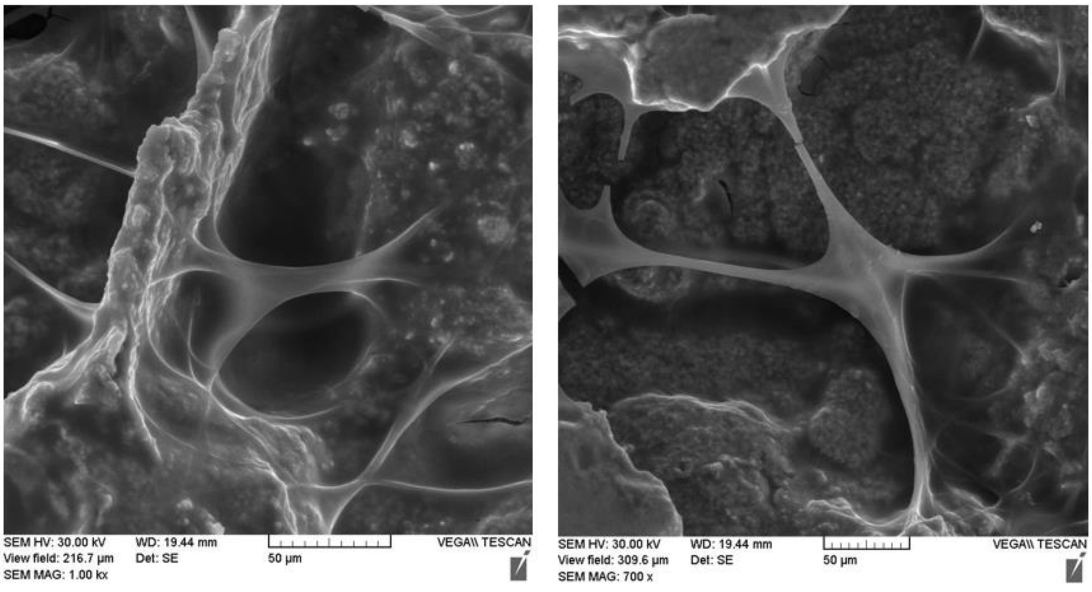

Fig. 10. SEM micrograph of MG-63 cells attached to the scaffold's surface using: (a) Mg and (b) Carbamide as space holders after 6 h**.**

It has been also reported by Itala et al [34] that bone ingrowth occurs even in pore sizes under 100 μm. It should be noted that there is difference between the effects of pore size on the in vitro and in vivo essays. In fact the results obtained from these two essays should be distinguished[35] . Generally during in vitro experiment, high fluid velocity gives cells less time to attach on the surface of the scaffold. Therefore smaller pore sizes presents better cell seeding whereas due to high fluid of oxygen and nutrition through in vivo essay larger pore sizes improves bone tissue regeneration[35] . It has been reported that cells preferred to spread on both sides of the large gaps between the particles to bridge easily. Such attached cells provide closer surfaces for new born cells to attach on them whereby bridging the gap will be much easier, in agreement with the hypothetical mechanism presented by Chen et al[36]. The results of MTT assay after 7-day incubation are illustrated in Fig. 11. The essay was carried out three times for each scaffold and mean values were used for comparison. The samples did not show any toxic effects. The fabricated sample used carbamide showed roughly 80% of viability while the produced FGS employed Mg as a spacer illustrated more than 85% of proliferation rate compared to control sample. The difference between the values obtained from scaffolds could be due to the type of space holder used. Magnesium solubility in titanium is expected to be negligible. In addition, it improves osteoconductivity of scaffolds if it remains and does not have any bad effect on biomedical applications[25] whereas incomplete removal of carbamide through debinding process may form toxic compounds in small quantities that really affect proliferation and cell viability. Another important factor that influences cells behavior is pore shapes. As can be seen in Fig. 5, pores are mostly circular in contrast to Fig.3, which shows irregular type of pores as a consequence of deforming of carbamide during compacting. According to Van Bael et al[37] , it seems that pores with more corners (closer to circular type) give more chance to cells to bridge. However, the pore occlusion could result in cell death inside the scaffold due to lack of nutrient and oxygen supply in agree with the findings of Melchels et al[38] . Thus the use of functionally graded scaffolds, which combine small pores in the seeding direction for initial cell attachment and large pores to delay pore occlusion and also provide higher medium diffusivity is suggested[37]. The produced FGS used magnesium as a space holder in this research suggests small circular pores inside increasing the cell attachment and large interconnected pores outside leading to higher average fluid velocities and less pore occlusion.

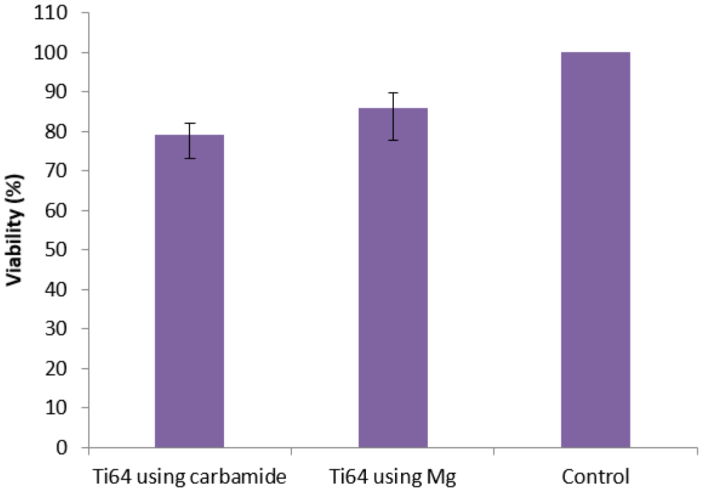

Fig. 11. MG-63 cell proliferation determined by MTT of porous samples after 7 days.

Conclusions

Ti-6Al-4V FGSs using Mg and carbamide as space holder were fabricated by powder metallurgy technique. The following conclusions are drown.

1. Powder metallurgy route is a low cost and simple method to produce and also control the pore characterizations of fabricated FGSs.
2. Pores shapes and sizes are more regular in FGSs having magnesium as a space holder while agglomeration of carbamide causes formation of heterogenous pores.
3. Compressive strength (47-160MPa) and Young's moduli (25-75) of the fabricated FGSs using Mg as a space holder seem to be appropriate for bone replacement application.
4. Both groups of samples showed considerable cell attachment. Cells preferred to bridge on both sides of the large gaps between the particles.
5. Although the fabricated sample with Mg as a spacer presented better cell proliferation, no difference in cell attachment was seen between both groups.

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