

Poly (Lactic-Co-Glycolic)/Nanostructured Merwinite Porous Composites for Bone Tissue Engineering: Structural and in Vitro Characterization

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ABSTRACT

Several characteristics of a novel PLGA/Merwinite scaffold were examined in the present study to evaluate the possible applications in bone tissue regeneration. Physical and mechanical properties, as well as degradation behavior and in vitro bioactivity of porous scaffolds produced by solvent casting and particle leaching technique were also characterized. Results showed that incorporation of merwinite particles into the porous polymer structure had a significant effect on cell viability in such a way that cell densities increased by increasing the merwinite content in the scaffolds after 3 and 7 days of culture. In contrast, mechanical analysis showed that the presence of merwinite had an adverse effect on the compressive strength of porous structures, due to the lack of formation of a chemical bond at the polymer-ceramic interface and non-homogenous distribution of the ceramic particulates through the matrix. Incorporation of the merwinite particles caused about 35% decrease in the compressive strength in samples containing 30 wt% merwinite, compared to pure PLGA porous scaffolds.

1. Introduction

There is an increasing need for development of bone tissue engineering due to various clinical bone diseases like bone infections, large bone defect for tumors removal, and bone loss by trauma [1]. Although there are many published reports regarding the design and application of new materials and constructs, there is still a need for the development of reliable and sustainable biocompatible structures to respond like natural bone in biological environment [1, 2]. One approach to mimic the natural bone

structure and its functions is to produce a temporary 3D porous composite material, made of a polymer matrix and ceramic particles, like the structure of natural human bones [3, 4]. In principle, a biodegradable polymer matrix with good degradation rate, sufficient mechanical strength and optimized pore architecture is desired. The osteoconductivity of the scaffold should also be considered as a key factor to make the adhesion and migration of the osteoprogenitor cells possible on the scaffold walls, and to facilitate the formation of new

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bone [5]. The latter could be achieved by incorporation of bioactive ceramic particles into the polymer matrix to improve osteoconductivity [6] and control the degradation rate of the scaffold and its by-products [5].

Synthetic polyesters like polyglycolide (PGA), polylactide (PLA), and their copolyesters poly(lactide-co-glycolide) (PLGA) have shown good biocompatibility and have been used as scaffold materials [7, 8]. However, application of these polymer materials is restricted due to several problems such as lack of bioactivity and hydrophilicity. Also, degradation of these polymers in biological environment leads to a decrease in the pH value because of releasing acidic by-products [9]. To overcome the mentioned difficulties, many researchers suggested using of bioactive ceramic particles like HA [10], β -tricalcium phosphate (β -TCP) [11] and bioactive glass [12] incorporated as the second phase dispersed in the polymer matrix. These ceramic components can improve the bioactivity of scaffold and control the pH value as well. The latter is related to releasing of the Si and Ca ions, which neutralizes the acidic by-products of degradation and stabilizes the pH value efficiently [13, 14].

Merwinite is a component of CaO-SiO₂-MgO system and possesses good bioactivity and biocompatibility like the other compounds of this ternary system. Previous researches have shown that merwinite stimulates apatite formation after soaking in simulated body fluid (SBF) and in-vivo studies revealed that merwinite better induces bone formation in comparison with HA and promotes osteogenesis more effectively [15-18]. The rate of the new bone formation is faster in bone defects filled by merwinite compared with those filled by HA granules [17].

In our previous study (hereafter referred to as Paper I) [19], a porous PLGA/merwinite composite scaffold was fabricated by solvent casting and particle leaching method. Preparation, morphology, and degradation patterns of the composite scaffolds were presented in Paper I. In the current study, chemical and mechanical properties of PLGA/merwinite composite scaffolds, as well

as cellular response are evaluated extensively by considering the degradation data presented in Paper I.

2. Experimental

2.1. Preparation of the PLGA/Merwinite composite scaffolds

Composite scaffolds were prepared by the method described in Paper I. In summary, 10 (w/v)% solution of PLGA (75:25, Boehringer Ingelheim) in dichloromethane (Merck) was prepared at room temperature, while continuously being mixed using a magnetic stirrer till the transparent solution was made after 60 minutes. NaCl particles (Merck) were sieved to 180-250 meshes and incorporated into the solution with PLGA:NaCl mass ratio of 1:9 to provide proper porosity in the final structure. Nanostructured merwinite powder was synthesized by sol-gel method as described in a previous study [15]. Certain amounts of merwinite powder (10, 20 and 30 wt%) were added to the PLGA solution followed by continuous stirring for 30 minutes to disperse merwinite and NaCl particles homogeneously. The resulting pastes were casted into cylindrical Teflon molds and dried at room temperature for 24 hours followed by 48 hours in vacuum oven at 60 °C for complete removal of solvents. Samples were immersed in deionized water for 48 hours to leach the progenes. Used water was replaced by fresh water every 6 hours during the leaching process to improve the leaching efficiency. Finally, the samples were freeze-dried for 48 hours to remove the excess water. Control samples (pure PLGA) were also prepared using the above mentioned procedures.

2.2. Structural and mechanical characterizations

Synthesized merwinite powder was analyzed by X-ray diffraction (XRD) using a Philips PW 3710 diffractometer. The apparent crystallite size of the powder was calculated from X-ray diffraction data through the Scherrer equation. Particle size analysis was carried out using Fritsch Analysete 22 apparatus to determine the mean value as well as the particles size distribution pattern of the synthesized powders. Fourier transformation infra-red spectroscopy

(FTIR) technique was used to characterize the scaffold materials with BRUKER Vector 33 spectrometer in the range of 400-4000 cm^{-1} . The compressive modulus and strength of the composite scaffolds were tested by HCR400-25 Universal Testing Machine (Instron, Zwick/Roell). The porosity of PLGA/Merwinite scaffolds was calculated by the following equation:

$$\text{Porosity} = (1 - \rho_{cs} / \rho_c) \times 100 \quad [1]$$

Where ρ_c is the density of initial composite with progen agent and ρ_{cs} is the density of composite scaffold. These two densities were measured using a pycnometer and determined by the gross weight and volume measurement of the specimens, respectively.

2. 3. Degradation behavior of the PLGA/merwinite composite scaffold

The degradation behavior of the PLGA/Merwinite porous scaffolds was investigated in Paper I. Since the relationship between chemical and mechanical properties of the synthetic composite scaffolds is discussed in the current study, we would extensively refer to the degradation data in Paper I. The degradation data, presented in Paper I, were collected based on the following method. In summary, in vitro degradation of porous scaffolds was carried out in PBS solution (Gibco#18912014) under pH 7.4 at 37°C. Weight loss during storage at 37°C in phosphate-buffered saline (pH 7.4) was determined for the scaffolds as well as PBS uptake. The buffer solution was changed every one week. The scaffolds were removed, rinsed with distilled water, dried at 37°C for measurement of weight loss every one week for a period of 8 weeks. The pH value of the samples in PBS was monitored every week. Degradation data for each of the sets were collected from three similar samples to obtain statistically meaningful data.

2. 4. Bone marrow obtaining

The use of the human bone marrow for research was approved by the Ethics Committee of Royan Institute (Tehran, Iran). Bone marrow was obtained from the patients who were candidate for stem cell

transplantation after myocardial infarction.

2. 5. MSCs isolation

For isolation of mesenchymal stem cells, bone marrow was mixed with phosphate buffer solution in equal ratio; the mixture was loaded on Ficoll (inno train) in 3:7 ratios and centrifuged at 1100 RPM for 5 min. The mononuclear cells were removed using pipette and the cells were supplemented with DMEM with 15% Fetal Bovine Serum (FBS, Gibco), 100 IU/ml penicillin (Sigma) and 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma, Germany) and plated. The cultures were incubated at 37 °C in humidified atmosphere of 5% CO₂. The first medium replacement was done on day 5 of culture and the subsequent changes of medium were performed every 3 days. With further successive subcultures, the MSC population was increased to a number sufficient to carrying out the next stages of the study.

2. 6. Bone Differentiation potential

To evaluate the osteogenic potential of the isolated cells, the medium of the passaged-3 culture was replaced by osteogenic DMEM medium containing 50 $\mu\text{g}/\text{ml}$ ascorbic acid 2-phosphate (Sigma; USA), 10 nM dexamethasone (Sigma; USA) and 10 mM β -glycerol phosphate (Sigma; USA) for 21 days at the end of which the cells were fixed with 10% formalin for 10 minutes and stained with alizarin red (Sigma; USA) for 15 minutes and observed by light microscopy.

2. 7. Three-dimensional culture

Before culture initiation, the cylindrical scaffold (1 cm diameter and 5 mm height) sterilized by 70% ethanol for 10 min and washed with PBS. Then, 5×10^5 passaged-3 MSCs were suspended in 50 μL DMEM medium and were placed on the top surfaces of the scaffolds located in wells of 12-well culture plate. Before the cultures were provided with medium, they were pre incubated at 37°C for 45 min during which the drop disappeared owing to its penetration into the scaffold porosity. The cultures were then provided with DMEM medium containing 15% FBS, 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin and incubated in an atmosphere of 5% CO₂ and

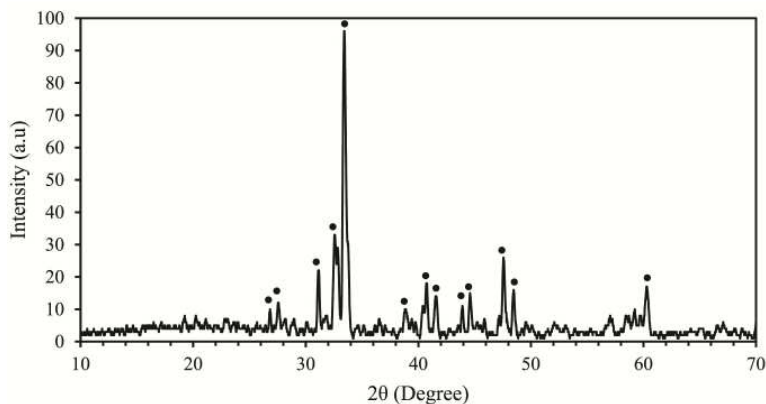


Fig. 1. XRD pattern of sol-gel synthesized merwinite ceramic

Temperature of 37°C.

2. 8. MTT assay (Cell proliferation)

Cell proliferation was analyzed by using MTT. This assay was based on the ability of live cells to reduce a tetrazolium-based compound, MTT, to a purplish formazan product. Briefly, the scaffolds were washed with PBS, transferred into new 12-well plates containing 5:1 ratio of media and MTT solution (5 mg/mL in PBS), respectively, and incubated for 2 h at 37°C. After 2 h, 500 μ l of DMSO was added to the MTT-treated wells and the scaffolds were washed extensively by pipetting up and down repeatedly to allow total color release. The absorbance of the supernatant was read with a microplate reader (BioTek EL x800, USA) at 570 nm. Cell population was determined through a standard curve that was established by using a known number of cells.

2. 9. Scanning Electron Microscopy (SEM)

The microstructure of the composite scaffolds was observed using Philips XL30 SEM. To examine the attachment and morphology of the loaded cells on the scaffolds, the cell loaded scaffold specimens from day-3 cultures were prepared for SEM observations. Briefly, MSCs-loaded scaffolds were fixed in 2.5% glutaraldehyde solution at 4°C for 24 h, followed by washing with PBS (phosphate buffer solution). The samples were then dehydrated sequentially with increasing concentration of ethanol (30%, 50%, 80% and 100%), coated with gold and visualized at 20KV accelerating voltage using a Vega II

XMU (TESCAN, Czech Republic) scanning electron microscope.

3. Result and discussion

3. 1. Structural and physical analysis

The XRD pattern of the synthesized merwinite powder is shown in Figure 1. According to the JCPDS database, sol-gel derived powder was composed of only the merwinite phase. Calculations of the apparent crystallite size using Scherrer equation showed that the merwinite powder had an average crystallite size of about 63 nanometers. Results of particle size analysis showed that the synthesized merwinite powder had a narrow size distribution and the mean particle size of merwinite powder was about 2.5 μ m and 95% of the synthesized powder particles were smaller than 4 μ m. The particle size distribution pattern has a direct effect on mechanical properties of scaffolds like compressive modulus and strength, and the distribution of applied forces would be strongly affected by the uniform size distribution of second phase particles [20].

The FTIR spectra of PLGA, other copolymers of PLGA and Merwinite have been reported in previous studies [21-24]. The FTIR spectrum of PLGA/Merwinite is presented in Figure 2. In Figure2, the sharp peak at 1751.25 cm^{-1} is attributed to carbonyl (C=O) absorption band of L-lactide and glycolide units in PLGA. Moreover, the peak at 1188.43 cm^{-1} can be corresponded to C-O stretching band of ester groups and the peaks at 1382.87 cm^{-1} , 1419.51 cm^{-1} could be assigned to -CH₃ group of PLA

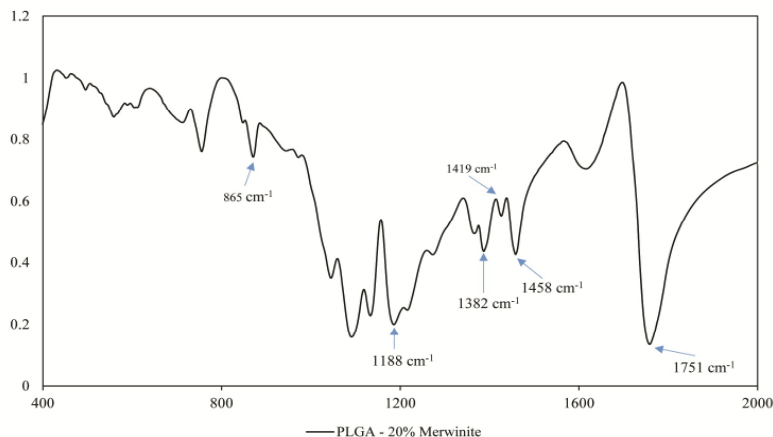


Fig. 2. FTIR spectrum of PLGA scaffold containing 20 wt% merwinite

Table 1. Absorption bands of PLGA- 20 wt% merwinite composite observed by IR spectroscopy.

PLGA		merwinite	
Absorption Bands (cm ⁻¹)	Assignment	Absorption Bands (cm ⁻¹)	Assignment
1751.25	C=O (stretching)	1039.56	Si-O-Si
1188.43	C-O (ester) (stretching)	594.03	Mg-O
1419.51	-CH ₂		
1458.30	as -CH ₃		
1382.87	s -CH ₃		
865.98	C-C		

And -CH₂ group of PGA, respectively. The peak at 865.98 cm⁻¹ is attributed to C-C bond. Finally, the peaks between 2700 and 3000 cm⁻¹ can be corresponded to the stretching band of -CH, -CH₂ and -CH₃ groups. The peak at 1039.56 cm⁻¹ is assigned to stretching vibrations of Si-O-Si bands in silicate groups of merwinite particles. There is also a band at 594.03 cm⁻¹ which is attributed to Mg-O. All the observed absorption bands of PLGA/Merwinite are reported in Table 1. Since no chemical shifts in the correspondent peaks of PLGA and merwinite were observed, it could be deduced that no chemical bond was formed between functional groups of PLGA and merwinite during processing of composite scaffolds.

Based on the data presented in Paper I, incorporation of ceramic particles into the polymeric matrix induced vast morphological changes in scaffolds microstructure. Figure 3 shows the microstructure of scaffolds with different ceramic contents. In general, by increasing the merwinite content, the pore walls became coarser and more irregular. Pore walls

became thicker by increasing the ceramic particle content up to 20 wt% and then an obvious decrease was observed in their thickness for samples containing 30 wt% of merwinite particles. Increasing the amount of ceramic particles would increase ceramic particles aggregation and local collapse of pore structures [25, 26]. This change in the pores structure could severely affect the mechanical behavior of scaffolds which is discussed in the following section. Measurements of scaffolds porosities showed no significant difference between scaffolds containing different amounts of ceramic particles, indicating the independency of scaffolds porosities from their ceramic content. The average amount of porosity in the composite scaffolds was 82 ±2%. This observation indicates that the porosity of scaffolds was totally dependent to the percentage of porogen used during the processing.

3. 2. Mechanical characterization

Results of mechanical characterization of the scaffolds are shown in Table 2. Calculations of

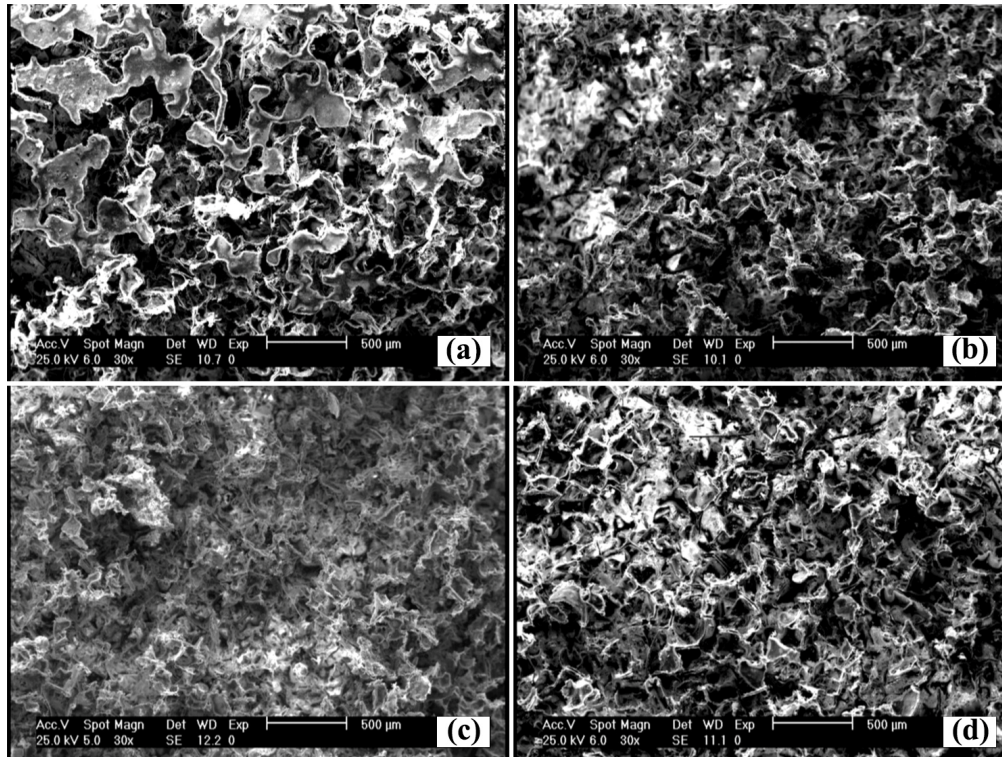


Fig. 3. SEM images of porous scaffolds containing different amounts of ceramic particles. (a) PLGA-10% merwinite (b) PLGA-20% merwinite (c) PLGA-30% merwinite (d) pure PLGA

Table 2. Mechanical properties of porous composite scaffolds containing different amounts of merwinite as the ceramic reinforcement.

Composition	Compressive Strength (MPa)	Compressive Modulus (MPa)
Pure PLGA	0.42 ± 0.04	9.3 ± 1.4
PLGA – 10 wt% merwinite	0.33 ± 0.02	7.5 ± 0.7
PLGA – 20 wt% merwinite	0.34 ± 0.01	7.5 ± 0.2
PLGA – 30 wt% merwinite	0.27 ± 0.02	6.6 ± 0.5

The compressive modulus and compressive strength of scaffolds containing different values of merwinite powder revealed that by increasing the ceramic content in porous scaffolds these two values decreased significantly. Two main factors could be considered for such a drastic decrease. First, the lack of formation of a strong bond between polymer matrix and ceramic particulates which induced crack growth at the ceramic/polymer interface and second, the heavy agglomeration of ceramic particles which resulted in non-homogeneous distribution of ceramic particles in the polymeric matrix. This inhomogeneity caused irregularity in microstructure of the pores which was previously observed by SEM

(Fig. 3). Collapse in pores structure could directly affect the mechanical strength of the porous scaffolds due to non-homogenous distribution of the applied forces. These two factors together with the significant difference between compressive modulus of merwinite and PLGA adversely affected mechanical properties of the porous scaffolds even by incorporating particles with narrow size distribution.

Comparison of mechanical data obtained from porous PLGA/nanostructured merwinite with those reported in the literature regarding the mechanical strength of natural bone (Table 3) indicates the significant difference between compressive strength of porous

Table 3. Mechanical properties of cortical bone [27].

Property	Value
Bending strength (MPa)	50–150
Fracture toughness (MPa m ^{1/2})	2–12
Young's modulus (GPa)	7–30
Compressive strength (MPa)	Longitudinal direction =70-280 Transverse direction = ~50

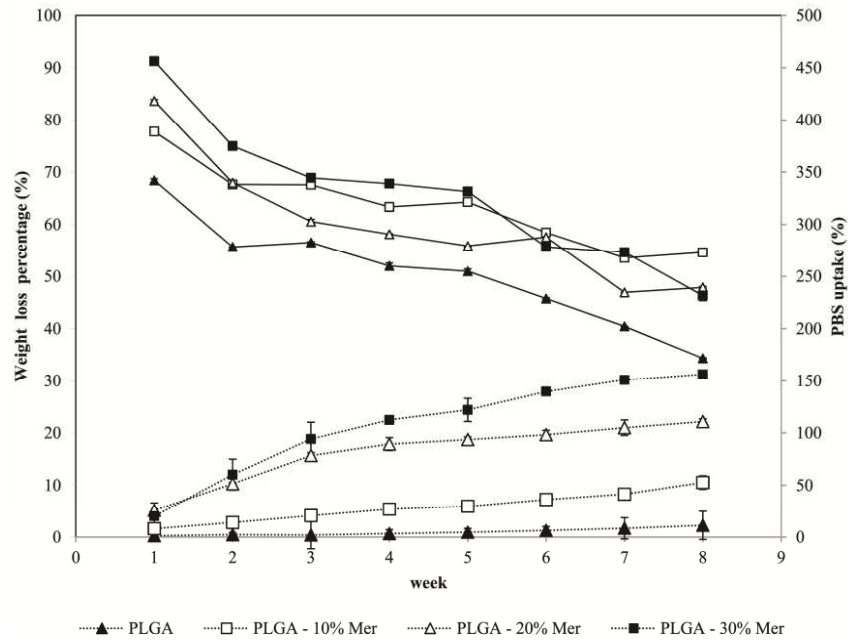


Fig. 4. Degradation behavior of porous PLGA scaffolds containing different amounts of merwinite. Upper dark lines represent the weight loss of scaffolds immersed in PBS for various time periods. Lower dotted lines refer to the PBS uptake in scaffolds incubated in PBS for various time periods (presented data were extracted from Paper I[19])

Scaffolds and natural bone. We speculate that the high porosity content of the scaffolds along with the lack of formation of strong bonding between polymer and ceramic phases are the main reason for such a drastic decrease. The possible application of PLGA/nanostructured merwinite composite scaffolds has to be restricted to non-load bearing sites due to their low mechanical properties.

3. 3. Degradation behavior

Degradation behavior of porous scaffolds plays an important role in the engineering process of a new tissue. The degradation rate of porous scaffolds affects cell vitality, cell growth, and even host response [28]. In vitro degradation behavior of porous composite scaffolds was studied in PBS solution in Paper I. The

previously published data collected on the scaffolds' weight loss and PBS uptake for samples immersed in PBS solution up to 8 weeks are combined and demonstrated in Figure 4. As can be seen, the rate of weight loss increased by increasing the ceramic content. This could be a result of hydrophilicity improvement in scaffolds which is a consequence of ceramic particles incorporation in the scaffold's structure. PLGA is often regarded as a hydrophobic biomaterial although it shows certain hydrophilicity because of the content of glycolic acid units in copolymer. This hydrophilicity facilitates water absorption in the polymer structure and leads to degradation by cleavage of ester bonds. Since the merwinite naturally possesses hydrophilicity characteristics, incorporation of

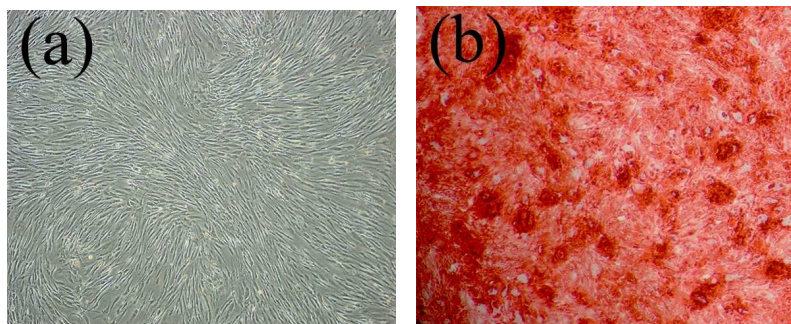


Fig. 5. MSCs isolation and bone differentiation. (a) Passaged- 3 human mesenchymal stem cells. (b) Alizarin red staining of passaged-3 mesenchymal stem cells indicating the cells ability to differentiate into a mineralized- matrix producing cells.

merwinite ceramic particles into polymeric scaffolds leads to enhanced water absorption, and thus, increase in degradation rate is observed. This correlation becomes clear by comparing the weight loss data with PBS uptake percentage in different incubation times (Figure4).

Changes in pH value of PBS during degradation of porous scaffolds revealed the effect of merwinite phase on the degradation behavior of PLGA scaffolds (Paper I). Immersion of pure PLGA scaffolds in PBS solution brought about a slight decrease in the pH value, while scaffolds containing merwinite particles caused an increase in the pH value. This increase had a direct relationship with the content of merwinite ceramic particles in such a way that by increasing the merwinite content, the reported pH value was higher. This phenomenon could be explained by considering the release of Ca and Mg ions as a consequence of merwinite particles degradation. After two weeks of incubation, a decrease in the pH value was observed for both samples with and without merwinite ceramic particles. This drop in the pH value might be considered as a function of the release of acidic by-products followed by polymer matrix degradation. By prolonging the incubation time, recorded pH in almost every sample group slightly decreased. However, there was a minor increase in the pH value in the fourth week of incubation which its magnitude proportionally increased by increasing the ceramic particles content. Ou et al. investigated the degradation behavior of merwinite ceramic and reported that the release of Ca, Mg, and Si ions caused a continuous

increase in the pH value from the first day of immersion in SBF and this increase lasted for 20 days and then reached a flat zone [18]. Wu et al. also conducted an in depth study on degradation behavior of the porous PLGA scaffolds and showed that in the initial stages of degradation a slight change in the pH value occurred and then after 8 weeks of immersion in PBS solution, massive release of acidic by-products caused a drastic decrease in the pH value [9]. By comparing the findings reported by these studies with the presented data in Paper I, it seems that the pattern of pH change during 8 weeks of incubation has been affected by the mutual release of ionic and organic compounds from ceramic and polymeric constituents, respectively. The role of ceramic particles in adjusting pH in early days of incubation can positively affect cell viability, which is discussed in the following section.

3. 4. Cellular response

3. 4. 1. MSCs isolation and characterization

Although most blood cells had already been removed from the bone marrow using Ficoll gradient centrifugation, some hematopoietic cells were still present in primary cultures. These cells were gradually eliminated as the primary cultures passed. The cultures appeared to be purified after three successive subcultures (Figure5a). The MSCs from passage -3 were used for evaluation of differentiation potential to osteoblast cells. The results showed that the MSCs have a good potency for differentiation to osteoblast lineage and the Ca^{+2} precipitation stained with alizarin red (Figure5b).

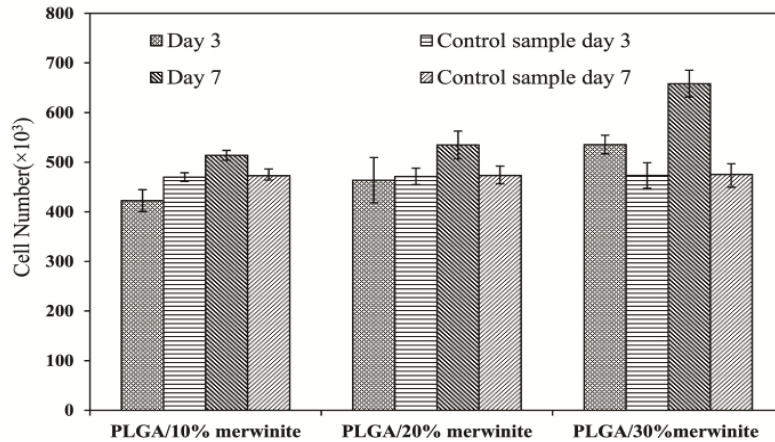


Fig. 6. MSCs proliferation in PLGA/merwinite composite scaffolds with different percentages of ceramic component.

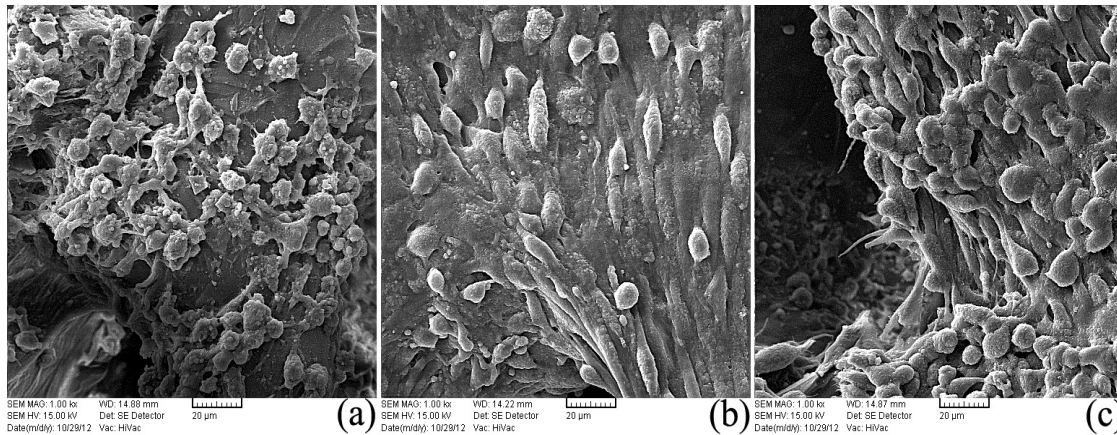


Fig. 7. SEM images of 3D cultures. (a) MSCs on PLGA/10 wt% merwinite, (b) MSCs on PLGA/20 wt% merwinite and (c) MSCs on PLGA/30 wt% merwinite. Cellular density seems to be much higher on composite scaffold containing 30 wt% merwinite.

3. 4. 2. MSCs attachment and proliferation on scaffolds

Cell viability and proliferation on scaffolds perused by MTT assay. This assay was based on the ability of live cells to reduce a tetrazolium-based compound, MTT, to a purplish formazan product. The results showed the number of cells in scaffolds containing 30 %wt merwinite has significantly increased compared to the scaffolds with 10 %wt and 20%wt of merwinite powder. The presented data indicate that the higher percentage of merwinite in combination with PLGA created more suitable environment for cells survival (Figure 6). The cells attachment and proliferation were also observed by SEM and

depicted in Figure7. It seems that the scaffolds containing higher percentage of merwinite prepared more suitable environment for cells attachment/proliferation. The number of attached/proliferated cells increased by increasing the merwinite content of corresponding scaffolds, suggesting that, presence of merwinite enhanced the bioactivity after seven days of culture.

4. Conclusion

Results of the current study showed that by incorporation of merwinite ceramic particles into porous PLGA scaffolds prepared by solvent casting and particle leaching, mechanical characteristics of scaffolds changed

drastically while no chemical bonds were observed between polymer and ceramic constituents. Degradation behavior of the porous scaffolds, which was presented previously in Paper I, revealed the positive effect of the merwinite content on controlling the pH of medium which, in turn, improved the cellular viability in scaffolds containing ceramic phase. MTT assay and SEM observations showed that by increasing the merwinite contents of porous scaffolds, more suitable environment was developed for proliferation and attachment of MSCs in early days of culture.

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