

Preparation and Characterization of Polyhydroxybutyrate Scaffolds to be Used in Tissue Engineering Applications

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Abstract

Polyhydroxyalkanoates (PHA) are good alternatives on account of biocompatible and biodegradable properties to produce materials as scaffolds for engineered tissues. Polyhydroxybutyrate (PHB) which is a member of polyhydroxyalkanoate family have been widely used as a biomaterial for *in vitro* and *in vivo* studies due to its unique properties such as improved flexibility and processability. In this study polyhydroxybutyrate scaffolds were prepared for tissue engineering applications. In order to improve cell attachment on the scaffolds they were modified. During the modification three different immunologically inactive compounds, polyethylene glycol (PEG), 2-hydroxyethyl methacrylate (HEMA) and ethylenediamine (EDA) were used in radio frequency glow discharge (RFGD) plasma polymerization system. Morphological evaluations were obtained by using scanning electron microscopy. Obtained results showed high and interconnected porosity. *In vitro* weight loss profiles of the scaffolds were investigated by using gravimetric method and found to be influenced by PHB concentration used in the preparation of scaffolds. Their biological promotion of activities including cell attachment, morphology and proliferation on L929 mouse fibroblast cells were examined and cytotoxicity tests were performed at the last part of the study.

Key Words: Polyhydroxybutyrate, scaffold, tissue engineering, morphology, glow discharge.

INTRODUCTION

Tissue engineering is a multidisciplinary field which combines the principles of engineering, chemistry and biology to restore the functionality of damaged tissue/organ through repair or regeneration [1]. One common approach is to isolate specific cells through

a small biopsy from a patient to grow them on a three-dimensional scaffold under controlled culture conditions. Afterward the construct is delivered to the desired site in the patient's body to direct new tissue formation into the scaffold that can be degraded over time [2-4]. An alternative approach is to implant scaffolds for tissue ingrowth directly *in vivo* to stimulate and direct tissue formation *in situ* [5,6]. The advantage of this approach is the reduced number of operations needed, resulting in a shorter recovery time for the patient. Therefore one of the key issues in tissue engineering is the development of suitable biodegradable materials and scaffolds for

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seeding cells and for the subsequent growth of tissues [7]. The requirements of scaffold materials for tissue engineering are manifold and extremely challenging [8]. The scaffold should (a) have interconnected porosity of appropriate scale to allow tissue integration and vascularisation, (b) possess appropriate surface chemistry to favour cellular attachment, differentiation and proliferation (c) have suitable mechanical properties to match the intended site of implantation and handling, (d) be produced from material with controlled biodegradability or bioresorbability so that tissue will eventually replace the scaffold, (e) should not induce any adverse response and, (f) be easily fabricated into a variety of shapes. Potential materials with these characteristics include natural polymers, synthetic polymers, ceramics, metals, and combinations of these materials [9].

Polyhydroxyalkanoates (PHA) are polyesters produced by microorganisms under unbalanced growth conditions. They are generally biodegradable and thermoprocessable, making them attractive as biomaterials for applications in both conventional medical devices and tissue engineering [10]. Poly(3-hydroxybutyrate) (PHB), as a member of polyhydroxyalkanoates (PHA) family, has attracted much attention because of its biodegradation and excellent biocompatibility [11].

In this study, PHB scaffolds were fabricated through a freeze-drying procedure. Resultant scaffolds were modified by using glow-discharge technique with poly(ethylene glycol) (PEG), 2-hydroxyethyl methacrylate (HEMA) and ethylenediamine (EDA) to increase cell attachment and proliferation. Subsequently, they were characterized based on morphology and *in vitro* weight loss. Cell attachment, morphology and proliferation on the prepared scaffolds were investigated *in vitro* and cytotoxicity of scaffolds was measured by MTT assay.

EXPERIMENTAL

Materials

Poly[(R)-3-hydroxybutyrate] (PHB) was supplied in powder form by Fluka (Switzerland) and had a weight average molecular weight (M_w) of 540,000 g/mol. Chloroform was used as a solvent and obtained from Sigma (USA). Polyethylene glycol (Acros, Belgium, M_w =300 kDa), 2-hydroxyethyl methacrylate (Aldrich, USA) and ethylenediamine (Fluka, USA) were used for the modification of PHB scaffolds. [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium Bromide] (MTT) was purchased from Aldrich (USA). The growth medium consisting of Dulbecco Modified Medium (DMEM), supplemented with fetal calf serum (FCS), penicillin-streptomycin and trypsin-EDTA were purchased from Biological Industries (Israel).

Preparation of PHB Scaffolds

PHB scaffolds were prepared by using freeze-drying technique [12]. Briefly 0.025 g, 0.05 g and 0.1 g of PHB were dissolved in 5 ml of chloroform to prepare solutions of 0.5%, 1.0% and 2% (w/v), respectively. The resultant solutions were then poured onto petri dishes and frozen overnight at -80°C . Subsequently, they were freeze-dried overnight and porous scaffolds were obtained. Scaffolds were kept in a vacuum desiccator for further analysis.

Plasma Modification of PHB Scaffolds

PHB scaffolds were modified by the radio frequency glow discharge (RFGD) plasma deposition technique. Plasma modification system (Vacuum, Praha) was equipped with 13.56 MHz radio frequency generator. The plasma reactor was attached with vacuum pump for evacuation of reactor gas. The reactor was fed with monomer tank and argon gas during the process. For the modification polyethylene glycol (PEG), 2-hydroxyethyl methacrylate (HEMA) and ethylene diamine (EDA) were used. The scaffolds were

placed onto a stereofoam support deployed in the middle of the electrodes with 1 cm spaces between each of species. The argon gas was passed through the reactor at 0.1 mbar pressure in order to sweep away any reactive species like oxygen and nitrogen. Subsequently, the reactor was fed with coating compounds and the glow discharge initiated at power of 35 W. The plasma process lasted for 20 minutes and the argon gas was passed through the chamber again to sweep away any gaseous residue. The scaffolds were kept in vacuum for 10 minutes for the stabilization of the modification.

Morphological Evaluations

Morphological evaluations of scaffolds were investigated by scanning electron microscopy (JEOL, Japan). A small piece of scaffold was put onto the sample holder, coated with gold and then the SEM micrographs were taken in these studies.

In Vitro Weight Loss Studies

The scaffolds were weighed and then immersed in phosphate buffered saline solution (PBS, pH 7.4) at 37°C for 8 weeks with daily solution exchange and gentle shaking in order to determine their weight loss profiles. Samples were removed periodically, dried in a vacuum oven and their weight was recorded. The weight loss was calculated as:

$$\text{Weight Loss (\%)} = \frac{W_o - W_f}{W_o} \times 100$$

where W_o and W_f are the weights of the dried scaffolds before and after exposure to water, respectively. Five samples were tested for each point. The mean values were presented in the graphs.

Determination of Cellular Viability and Cytotoxicity (MTT Assay)

MTT assay is a simple colorimetric assay to measure cell cytotoxicity, proliferation and viability [13]. Cytotoxicity effects of degradation yields of non-modified and modified PHB scaffolds were investigated on cultured L929 mouse fibroblast cells. The scaffolds were interacted with the cell culture medium and incubated for 5 days at 37°C. The cells were plated in 96-well culture plates at 17×10^3 cell/well. They were cultured in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin in humidified 5% CO₂ at 37°C. Following the incubation, mediums were removed and added to the medium interacted with the scaffolds. At the end of 24 h, mediums were removed and 100 µl of fresh medium and 13 µl of MTT solution (5 µg/ml, diluted with RPMI 1640 without phenol red) were added to the each well. Incubation was allowed for another 4 h in dark at 37°C. Mediums were removed and 100 µl/well isopropanol-HCl (absolute isopropanol containing 0.04M HCl) solution was added to dissolve formazan crystals. The wells were read at 570 nm on an ELISA plate reader and percentage of viability calculated. Cell viability was defined as 100% for MTT assay control.

The morphological characteristics of the cells cultured onto the scaffolds were also investigated. L929 mouse fibroblast cells were seeded on the scaffolds and incubated at 37°C for 4 days. Following the incubation, scaffolds were washed 3 times with PBS. Loosely adherent or unbound cells were removed and scaffolds were dyed with giemsa stain. Morphology of bound cells were observed by optical microscopy.

RESULTS AND DISCUSSION

Morphological Evaluations

Figure 1 shows the cross-section and surface morphology of polyhydroxybutyrate scaffolds. The cross-section of scaffolds showed an interconnected network pore configuration as can be seen from Figure 1a. Also as shown in Figure 1b, a homogeneous and highly porous structure was observed by SEM. Observed well-interconnected pore network structure and large surface area are necessary for cellular attachment and vascularization [14].

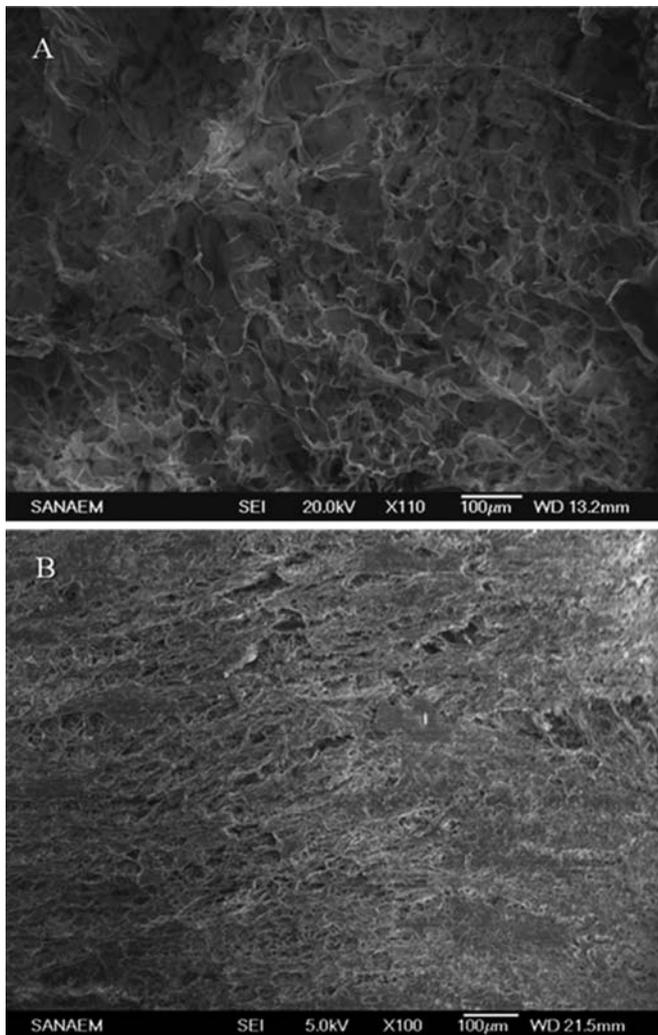


Figure 1. Scanning electron micrographs of (a) cross-section and (b) surface of polyhydroxybutyrate scaffolds.

In Vitro Weight Loss Studies

Scaffolds which were prepared with three polymer concentrations (0.5, 1.0 and 2.0% (w/v)) were selected for this study. They were exposed to

aqueous medium and their weight loss was measured at different time intervals for 8 weeks. The scaffolds exhibited progressive mass loss over the 8-week period. Obtained weight loss profiles were shown in Figure 2.

The rate of scaffold degradation was the slowest using polymer concentration of 2.0%. Under this condition, only 4% of the scaffold was degraded after 8 weeks of incubation. On the other hand, the rate of scaffold degradation was the fastest using a polymer concentration of 0.5%. 17 % of the scaffold had degraded after 8 weeks of incubation. PHB scaffolds made from 0.5% polymer had greater degradation than those made from higher solution concentrations of 1.0% and 2.0%.

As it is very well-known, the pore structure and morphology have definite effects on the degradation behaviors of the scaffolds made of the same raw material. Different porosities and pore sizes lead to different thicknesses of pore walls for porous scaffolds, which might turn out to affect the underlying degradation behaviors [15]. In our study we obtained different pore structure and morphology for PHB scaffolds made from different concentrations of polymer which led to different weight loss profiles.

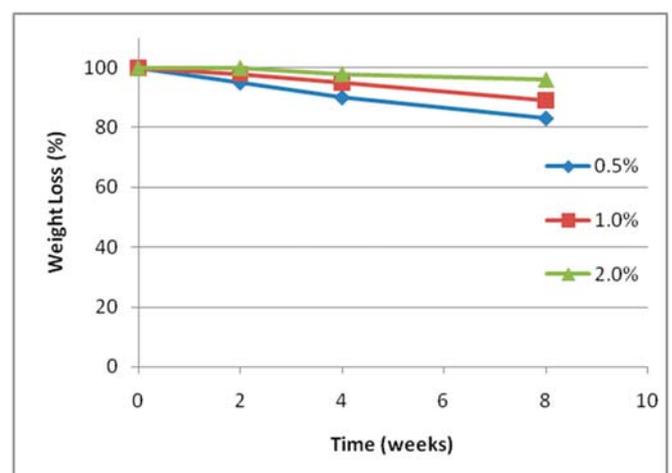


Figure 2. Percent weight loss versus time for PHB scaffolds made from different concentrations of polymer.

Table 1. Cell viability evaluated by MTT assays of modified and non-modified PHB scaffolds showing the influence of modification and polymer concentration on mouse L929 fibroblast cell line

	Absorbance	Cell Viability (%)
Control	0.480	100 ± 2
Non-modified PHB scaffold		
0.5%	0.440	91.6 ± 2
1.0%	0.480	100.8 ± 2
2.0%	0.472	98.3 ± 2
EDA modified PHB scaffold		
0.5%	0.438	91.2 ± 2
1.0%	0.466	97.0 ± 2
2.0%	0.460	95.8 ± 2
HEMA modified PHB scaffold		
0.5%	0.426	88.7 ± 2
1.0%	0.460	95.8 ± 2
2.0%	0.469	97.7 ± 2
PEG modified PHB scaffold		
0.5%	0.437	91.0 ± 2
1.0%	0.466	97.0 ± 2
2.0%	0.474	98.7 ± 2

Cytotoxicity Assay (MTT)

To observe the cytotoxic effects of the modified and non-modified PHB scaffolds on L929 mouse fibroblast cell lines *in vitro*, MTT test was performed. MTT test results were given in Table 1 and Figure 3. It was observed that the viability of L929 cells decreased with the decrease in the PHB concentration due to the fast degradation in the case of lower polymer concentration. The results obtained with the modified PHB scaffolds indicate that no inherent toxicity can be attributed to the modifications. As can be seen from Table 1 and Figure 3, the cell viability for the non-modified and modified PHB scaffolds at 2% concentration are 98.3%, 95.8%, 97.7%, 98.7% after 5 days, which means that the cytotoxicity of the 2% PHB scaffolds on the L929 cells is not significant.

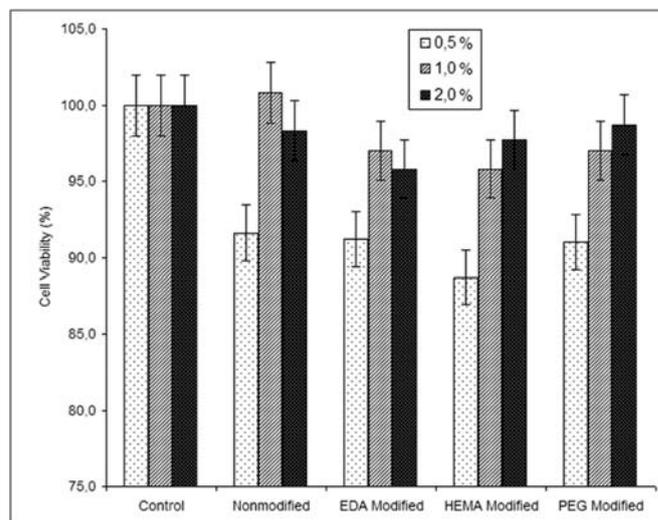


Figure 3. Cell viability evaluated by MTT assays of modified and non-modified PHB scaffolds showing the influence of modification and polymer concentration on mouse L929 fibroblast cell line.

Cell Attachment and Morphology

To observe the morphological characteristics of the cells cultured onto the scaffolds and cell attachment-spreading, L929 mouse fibroblast cells were seeded on the scaffolds and incubated at 37°C for 4 days. After the incubation, scaffolds were washed 3 times with PBS. Loosely adherent or unbound cells were removed and scaffolds were dyed with giemsa stain. The bound cells were investigated by light microscopy. Obtained photographs were given in Figure 4 and 5. The data implies that the modified PHB scaffolds induces cell attachment and proliferation as seen in Figure 4. L929 fibroblast cells showed a significant attachment to the scaffold at day 4 as compared to non-modified scaffolds.

The cells were also investigated morphologically. Because the biocompatibility of biomaterials is extremely closely related to cell behavior on contact with them and particularly to cells adhered to their surface [16]. Cell nuclei and cytoplasm were observed clearly as shown in Figure 5. Obtained images indicated that the prepared scaffolds had good biocompatibility.

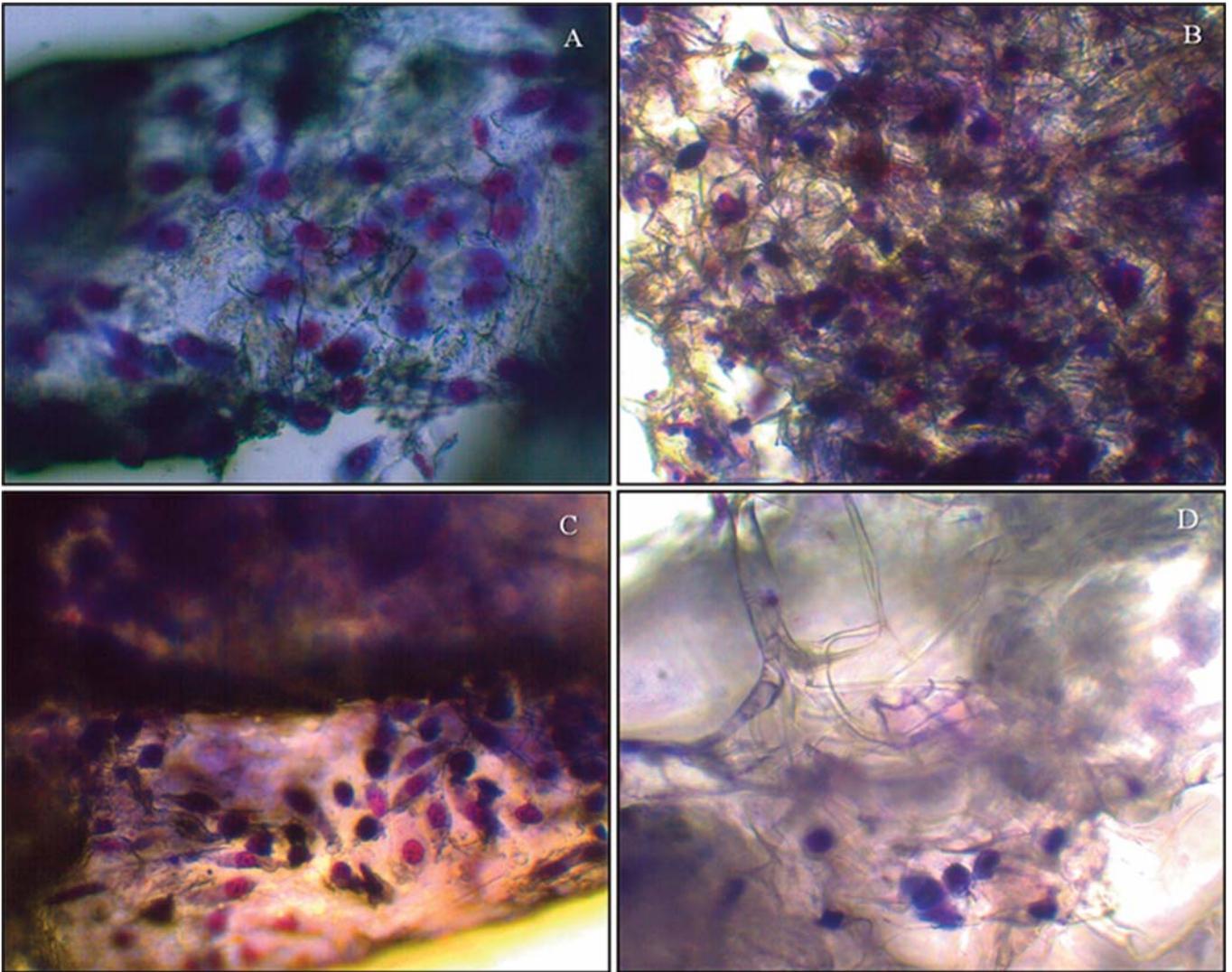


Figure 4. Optical images showing mouse fibroblast cell attachment on (a) HEMA-modified (b) EDA-modified (c) PEG-modified (d) Non-modified PHB scaffolds. Cells were stained by Giemsa stain.

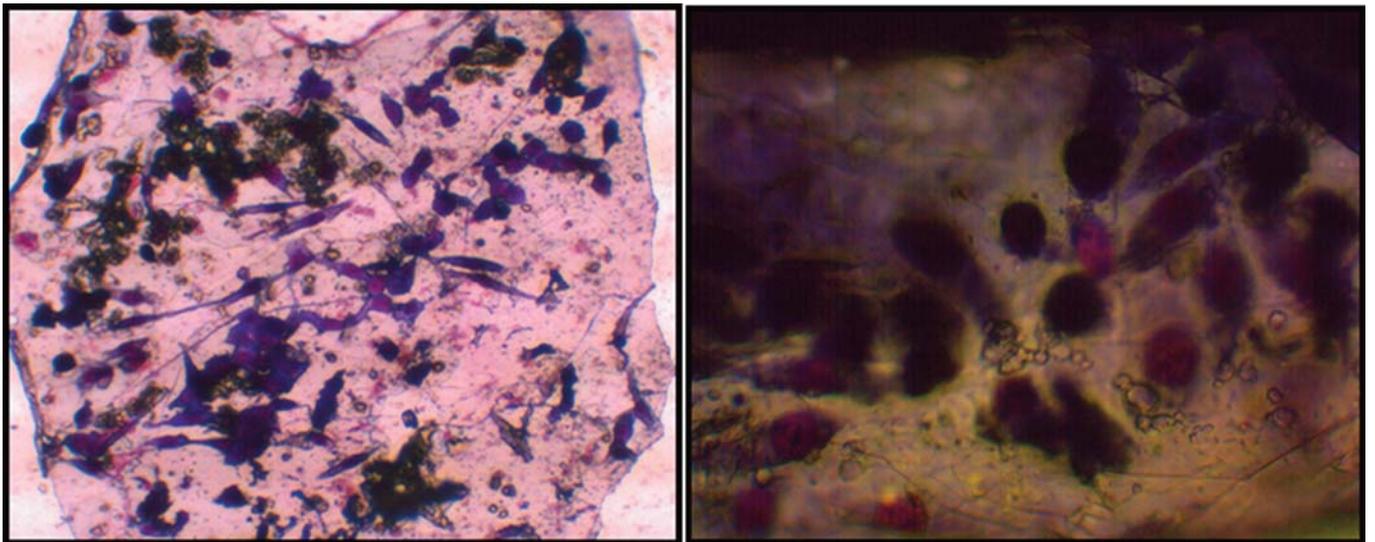


Figure 5. Optical images showing attached cell morphology on the scaffolds regarding biocompatibility.

REFERENCES

1. Yarlagadda, P.K., Chandrasekharan, M. and Shyan, J.Y.M., Recent advances and current developments in tissue scaffolding, *Bio-Medical Materials and Engineering*, 15 (2005) 159-177.
2. Williams, D., Benefit and risk in tissue engineering, *Mater Today*, 7 (2004) 24–29.
3. Mano, J.F., Sousa, R.A., Boesel, L.F., Neves, N.M., Reis, R.L., Bioinert, biodegradable and injectable polymeric matrix composites for hard tissue replacement: state of the art and recent developments, *Compos. Sci. Technol.*, 64 (2004) 789–817.
4. Shin, H., Jo, S., Mikos, A.G., Biomimetic materials for tissue engineering, *Biomaterials*, 24 (2003) 4353–4364.
5. Drotleff, S., Lungwitz, U., Breunig, M., Dennis, A., Blunk, T., Tessmar, J., Biomimetic polymers in pharmaceutical and biomedical sciences, *Eur. J. Pharm. Biopharm.*, 58 (2004) 385–407.
6. Suchanek, W., Yoshimura, M., Processing and properties of hydroxyapatite-based biomaterials for use as hard tissue replacement implants, *J. Mater. Res.*, 13 (1998) 94–117.
7. McIntire, L.V. (Ed), WTEC Panel on Tissue Engineering Research: Final Report, Academic Press, San Diego, 2003.
8. Sachlos, E., Czernuszka, J.T., Making tissue engineering scaffolds work. Review on the application of solid freeform fabrication technology to the production of tissue engineering scaffolds, *European Cells and Materials*, 5 (2003) 29-40.
9. Whang, K., Healy, E., Elenz, D.R., Nam, E.K., Tsai, D.C., Thomas, C.H., Nuber, G.W., Glorieux, F.H., Travers, R., Sprague, S.M., Engineering bone regeneration with bioabsorbable scaffolds with novel microarchitecture, *Tissue Eng.*, 5 (1999) 35-51.
10. Chen, G.Q., and Wu, Q., The application of polyhydroxyalkanoates as tissue engineering materials, *Biomaterials*, 26 (2005) 6565–6578
11. Chen, L.J., Wang, M., Production and evaluation of biodegradable composites based on PHB–PHV copolymer, *Biomaterials*, 23 (2002) 2631–2639.
12. Hsu, Y.Y., Gresser, J.D., Trantolo, D.J., Lyons, C.M., Gangadharam, P.R.J., Wise, D.L. Effect of polymer foam morphology and density on kinetics of in vitro controlled release of isoniazid from compressed foam matrices, *J Biomed. Mater. Sci.*, 35 (1997) 107-116.
13. Mansur, H.S., Costa, H.S., Nanostructured poly(vinyl alcohol)/bioactive glass and poly(vinyl alcohol)/chitosan/bioactive glass hybrid scaffolds for biomedical applications, *Chemical Engineering Journal*, 137 (2008) 72–83.
14. Jiang, L., Li, Y., Wang, X., Zhang, L., Wen, J., Gong, M., Preparation and properties of nano-hydroxyapatite / chitosan / carboxymethyl cellulose composite scaffold, *Carbohydrate Polymers*, 74 (2008) 680–684.
15. Wu, L., Ding, J., Effects of porosity and pore size on in vitro degradation of three-dimensional porous poly(D,L-lactide-co-glycolide) scaffolds for tissue engineering, *Journal of Biomedical Materials Research*, 75 (2005) 767-777.
16. Gong, S., Wang, H., Sun, Q., Xue, S., Wang, J., Mechanical properties and in vitro biocompatibility of porous zein scaffolds, *Biomaterials*, 27 (2006) 3793–3799.