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Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) based tissue engineering matrices

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In this study, the aim was to produce tissue-engineered bone using osteoblasts and a novel matrix material, poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV). In order to prepare a porous PHBV matrix with uniform pore size, sucrose crystals were loaded in the foam and then leached leaving pores behind. The surface of the PHBV matrix was treated with rf-oxygen plasma to increase the surface hydrophilicity. SEM examination of the PHBV matrices was carried out. Stability of PHBV foams in aqueous media was studied. The pH decrease is an indication of the degradation extent. The weight and density were unchanged for a period of 120 days but then a significant decrease was observed for the rest of the study. Osteoblast cells were then isolated from rat bone marrow and seeded onto PHBV matrices. The metabolization and proliferation on the foams was determined with MTS assay which showed that osteoblasts proliferated on PHBV. It was also found that cells proliferated better on large pore size foams (300–500 μm) than on the small pore size foams (75–300 μm). Production of ALP was measured spectrophotometrically. The present study demonstrated that PHBV matrices are suitable substrates for osteoblast proliferation and differentiation.
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Introduction

In cases where bone loss or damage occurs a need for supplemental bone source arises. This problem is generally solved by using autografts, allografts, xenografts, natural and synthetic organic and inorganic materials (i.e. coral, ceramics, polymers, etc.). Each of these cases has its own problems such as high cost, risk of bacterial or viral infection, donor shortage, donor-to-donor variation in quality, and slow vascularization in the defect area [1]. Bone tissue engineering could be a potential remedy for these problems. For a successful application of such engineered tissues, the development of biomaterials with suitable mechanical characteristics and degradation behavior is very important. These materials need to be prepared via molding, extrusion, polymerization, etc. *in situ* before seeding with cells to provide a good fit in the defect area and also minimize the loss of cells used in initial seeding [2].

Osteoconductive matrices used as scaffold should satisfy certain requirements. They should be designed to allow diffusion of nutrients to the transplanted cells and guide cell organization, attachment, and migration [3]. They should also be biodegradable and bioresorbable. In addition, scaffold materials should reduce immune response to allogenic cells. Pore size and porosity are other important parameters. The structures have to allow optimum cell invasion and new blood vessels. The mechanical properties of the material should match that of the tissue. Mechanical properties of scaffolds during degradation and bone regeneration must be studied. There is increasing evidence that changes in scaffold surface chemistry and topography alter cellular activity [4]. Therefore, surfaces need to be characterized or even altered to facilitate bone tissue regeneration.

Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) is an optically active polyester consisting of D-β-hydroxybutyric acid (PHB) as a repeating unit. PHB is produced by various strains of microorganisms such as *Bacillus megaterium*, glucose utilizing mutant of *Alcaligenes eutrophus*, etc., soil bacteria, estuarine microflora, blue green algae, microbiologically treated sewage. It is also a normal constituent of human blood. It is safe, nontoxic, biocompatible, biodegradable, easily and reproducibly processable. The related copolymers of PHB with β-hydroxyvaleric acid (PHBV) emerge as a new generation of PHB-based materials with more adjustable properties depending upon copolymer composition. Degradation rate of PHBV can easily be adjusted by changing the copolymer composition. PHB and PHBV matrices lose mass very slowly when

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compared to bulk-degrading poly(lactide-co-glycolide) systems. PHBV are semicrystalline polymers that have high degrees of crystallinity (60–80%). Crystallinity could influence the rate and the mechanism of degradation, the biocompatibility, distribution and entrapment of solutes (i.e. drugs, enzymes, etc.) in the structure and as a result influences the mechanism and kinetics of drug release. PHBV are known to be biocompatible [5, 6] and exhibit piezoelectricity [7]. Since electrical stimulation is thought to promote bone healing and repair, use of polymers have been proposed for use as bone screws, pins, and plates.

Rivard *et al.* [8] demonstrated that PHBV9 (9% 3-hydroxy valerate in the structure) sustained fibroblast cell proliferation rate similar to that observed in collagen sponges for up to at least 35 days. On the other hand, the PHBV materials kept their integrity during the culture period while the collagen foams shrank significantly. Moreover, the total protein production after 4 weeks in culture was found to be twice as high in the PHBV foam than in the collagen foam.

In the literature there are a number of studies about tissue responses to PHBV materials and their *in vivo* stability [9, 10]. It was found that porous PHBV materials are adequate as substrates for cell cultures. PHB and PHBV polymers were well tolerated by the tissue when implanted subcutaneously in mice. No acute inflammation, abscess formation or tissue necrosis was observed in tissues adjacent to the implanted materials. Mononuclear macrophages, proliferating fibroblasts and mature vascularized fibrous capsules were typical of the tissue response.

When PHBV was implanted in rabbit tibia as a carrier of antibiotics cefoperazone and sulbactam, there were no significant symptoms of chronic inflammation or toxicity as judged by histology, SEM, microbiology, and X-ray studies [11].

Biomechanical properties and biocompatibility (shown via histology) of PHBV composites were also studied *in vivo* [12]. Enhanced endosteal bone growth was observed for the hydroxyapatite/PHB (HA/PHB) composites whereas no periostal or endosteal activity was detected for the HA/glass/PHB (HGP).

Materials and methods

Preparation of the materials

PHBV8 (contains 8% by mol of hydroxy valerate, Aldrich Chem. Co., Milwaukee, MI, USA) solution (6%, w/w) in chloroform : dichloromethane (1 : 2, v/v) was prepared.

In order to obtain a highly porous matrix with uniform pore sizes, sieved sucrose crystals (75–300 or 300–500 μm) were used. PHBV solution (6%, w/w) was poured onto the sucrose crystals until they were submerged which was then air-dried. The resultant foam was dialyzed for 2 days to remove all the sucrose, creating a porous PHBV matrix. This was frozen at − 70 °C and lyophilized in a freeze dryer (Labconco Freeze Dry, Missouri, USA, Model 78680). Matrices were cut into discs of 7 mm diameter and 1.9 mm thick.

The surface of the porous PHBV matrices were then treated with oxygen rf-plasma (Advanced Plasma System, Inc., USA) at 50 W for 10 min, 50 W for 20 min, 100 W for 10 min, and 100 W for 20 min, in order to make the surfaces more hydrophilic. Finally, the scaffolds were sterilized by exposure to gamma radiation (25 kGy).

Scanning electron microscopy (SEM)

Surfaces of the untreated and unloaded (UT); small sucrose (SS) loaded (75–300 μm), large sucrose (LS) loaded (300–500 μm), and oxygen plasma treated large sucrose loaded (O_2-LS) PHBV (6%, w/w) were all coated with gold and SEM was carried out in a JEOL (Japan, Model JSM 6400) system to observe the surface characteristics, pore sizes and the pore distribution of the matrix materials.

Aging in phosphate buffer

For the degradation study, UT and LS PHBV (6%, w/w) foams were used. The discs were put into 50 mL Corning flasks containing phosphate buffer (20 mL, pH 7.4, 10 mM, 0.09% sodium azide) and incubated at 37 °C for 0.5, 1, 2, 4, and 6 months. Aliquots from each sample were collected at different time points to measure the pH change of the medium (phosphate buffer). At the end of the period, the discs were freeze-dried for 8 h and weighed. All measurements were expressed as means ± standard deviation (sd) relative to the initial values.

Isolation and culture of osteoblast

Stromal osteoblastic cells were obtained from the marrrow of young adult male (6 weeks old, 150–170 g) Wistar rats. Following euthanasia by diethyl ether inhalation, femora were aseptically excized, cleaned of soft tissue, and washed in Dulbecco's Modified Eagle medium (DMEM) containing 1000 units/mL penicillin and 1000 units/mL streptomycin. The metaphyseal ends then were cut off and the marrow flushed from the midshaft with 5 mL of primary media (DMEM containing 20% fetal bovine serum (FBS), 100 units/mL penicillin and 100 units/mL streptomycin), and collected in a sterile Petri dish. The bone marrow aspirate was then centrifuged at 400 g for 10 min. The resulting cell pellet was resuspended in primary media and plated in T-75 flasks and incubated in CO_2 incubator (SANYO, Japan, Model MCO 175). After the first passage, complete media containing DMEM supplemented with 20% FBS, 100 units/mL penicillin, 100 units/mL streptomycin, 10 mM β-glycerophosphate, 50 μg/mL L-ascorbic acid and 10 nM dexamethasone were added to promote the osteoblastic phenotype of marrow stromal cells. Complete medium was changed every other day.

Seeding of osteoblasts on PHBV foams

Osteoblast cultures were trypsinized and counted. They were diluted to yield 1×10^5, 5×10^5, 10×10^5 cells/mL in complete media. Aliquots of 20 μL of cell suspension were seeded onto the top of prewetted matrices placed in the wells of 24-well plate. The matrices were left undisturbed in an incubator for 6 h to allow the cells to

attach to the matrix. Then, 1 mL of complete medium was added to each well. Medium was changed every other day.

MTS assay for the proliferation of osteoblasts

Osteoblast cultures were trypsinized and counted. Different concentrations of the osteoblast cells $(1 \times 10^4,\ 2.5 \times 10^4,\ 5 \times 10^4,\ 10 \times 10^4,\ 15 \times 10^4,\ 20 \times 10^4,\ 30 \times 10^4,\ 40 \times 10^4,\ 50 \times 10^4)$ were seeded on 96 well Elisa plates and incubated for 6 h at 37 °C in a CO_2 incubator. MTS/PMS reagent (Promega, USA, 100 μL) was added to each well of the 96 well plate which was then incubated at 37 °C in a CO_2 incubator. Absorbance was determined at 490 nm using an Elisa Plate Reader (Molecular Devices USA, Model Maxline) and a calibration curve using different concentrations of osteoblast cells was plotted.

Osteoblast growth on PHBV foams with different pore sizes

Osteoblast seeded SS, LS and O2-LS (100 W, 10 min) PHBV foams were incubated for 7 days in CO_2 incubator. MTS/PMS reagent (100 μL) was added to each well of the 24 well plate and incubated at 37 °C in a CO_2 incubator. Absorbance was determined at 490 nm using an Elisa Plate Reader.

Osteoblast growth on PHBV foams with different surface treatments

In order to study the effect of extent of oxygen plasma modification of the foams on osteoblast growth inside the matrices, PHBV foams were treated with rf-oxygen plasma (50 W, 10 min; 50 W, 20 min; 100 W, 10 min; and 100 W, 20 min). Osteoblast seeded foams $(2.5 \times 10^5$ cells/mL$)$ were incubated for 7 days in a CO_2 incubator. MTS/PMS reagent (100 μL) was added to each well of the 24 well plate and incubated at 37 °C in a CO_2 incubator. Absorbance was determined at 490 nm using an Elisa Plate Reader.

ALP activity

For the determination of the effect of the cell-substrate interaction on the production of alkaline phosphatase, cells (1.5×10^5 cells/polymer scaffold) were cultured for 7, 14, 21 and 29 days on a polymer scaffold. At each time point, the medium was removed from the wells and foams were washed three times with PBS. Then, they were freezed and thawed three times. Upon thawing, the foams were homogenized with 1 mL Tris buffer (1 M, pH 8.0) and sonicated for 5 min on ice.

A volume of 20 μL of each sample was added to 1 mL of p-nitrophenyl phosphate solution (16 mmol/L) (Diagnostic kit 245, Sigma) at 30 °C and maintained for 2 min. p-Nitrophenol was produced in the presence of alkaline phosphatase and was measured with a UV Visible spectrophotometer at 405 nm. The absorbance was measured at 1 and 2 min and the slope of absorbance vs. time plot was used to calculate the alkaline phosphatase activity. This experiment was performed in duplicate.

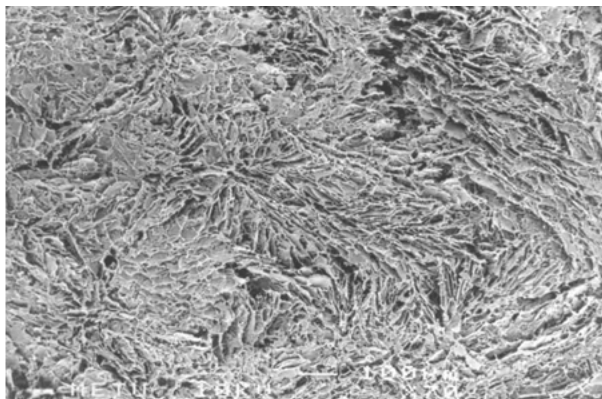

Figure 1 Scanning electron micrographs of untreated PHBV foams (6%, w/w) (no sucrose leaching to create pores), with chloroform : dichloromethane solution (1 : 2, v/v) of the polymer used in foam preparation (original magnification × 70).

Results

Materials characterization studies

Scanning electron microscopy (SEM)

It was found out from the SEM that untreated and unloaded PHBV had a more ordered, compact and smooth surface (Fig. 1). The amount of the pores was not sufficient for osteoblast growth.

After sucrose leaching, the number of the pores within the foams increased (Figs. 2 and 3). Two different sizes of the crystals were loaded inside the polymer (75–300 or 300–500 μm). Both the large and small size sucrose loaded foams allowed cell growth but it was observed that matrices with the larger pore size (300–500 μm) provided a better osteoblast growth environment.

In the case of oxygen rf-plasma treated PHBV foams, a more hydrophilic structure than that of the untreated ones was obtained. Before treatment, the surfaces of the foams appeared to be covered with a thin polymer film probably due to the solvent evaporation process used in foam preparation. The surface texture of the foams changed substantially upon treatment (Fig. 4), a change that could be attributed to etching of the very top (skin) layer of the membranes, exposing the pores created by removal of sucrose crystals.

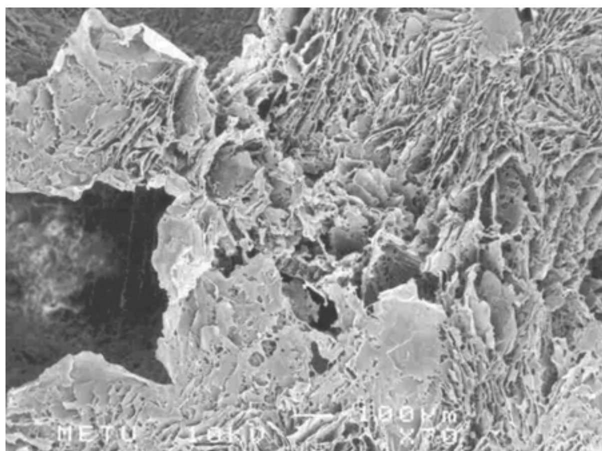

Figure 2 Scanning electron micrographs of untreated PHBV (6%, w/w) foams with sucrose-leaching (75–300 μm) (original magnification × 70).

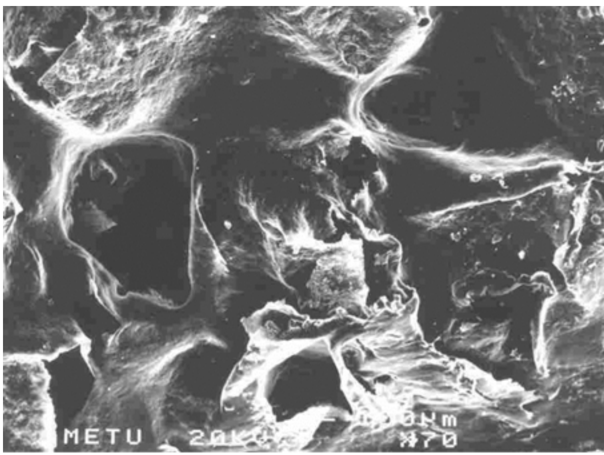

Figure 3 Scanning electron micrographs of untreated PHBV (6%, w/w) foams with sucrose-leaching (300–500 μm) (original magnification × 70).

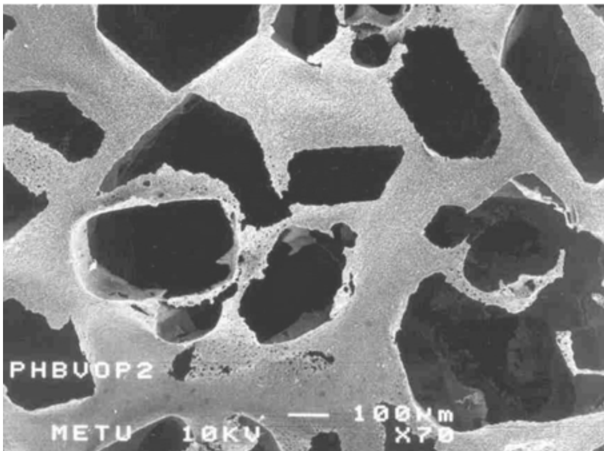

Figure 4 Scanning electron micrographs of rf-oxygen plasma treated (100 W, 10 min) PHBV (6%, w/w) foams with sucrose-leaching (300–500 μm) (original magnification × 70).

Aging in phosphate buffer

Matrices prepared using PHBV 6% led, in 180 days, to a pH decrease of 0.16 pH units when they were in unloaded (dense) foam form and 0.18 pH units when they were sucrose loaded PHBV foams. The pH decrease is an indication of the degradation, and its level is a measure of the extent of degradation and, therefore, the sample that leads to the largest pH drop would be the one that has degraded the most (Fig. 5). The data presented above, however, did not indicate any significant difference between the samples tested.

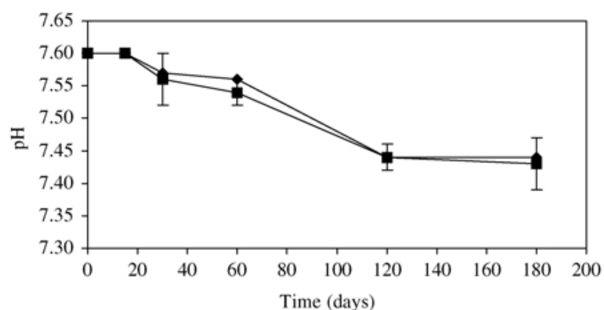

Figure 5 Change in the pH of the medium as an indication of degradation of unloaded and sucrose loaded PHBV (6%, w/w) foams. (◆) unloaded PHBV; (■) sucrose loaded PHBV.

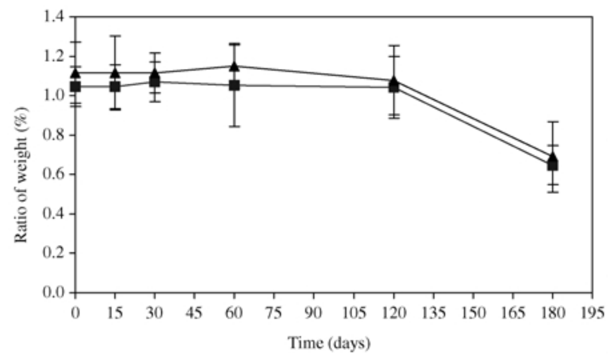

Figure 6 Degradation of PHBV foams; ratio of (initial to final) weight (%) vs. time graph for unloaded and sucrose loaded PHBV (6%, w/w). (◆) unloaded PHBV; (■) sucrose loaded PHBV.

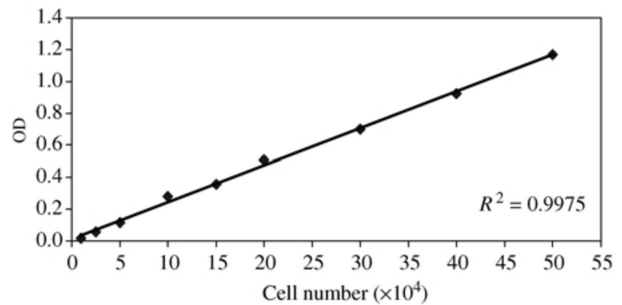

Figure 7 MTS calibration curve for osteoblast determination (an OD_{490} vs. cell number $\times 10^4$ plot).

After 120 days in the medium, weight loss started to be noticeable (Fig. 6). What is more significant is that there was no perceptible change in the first 120 days and then a 2-fold decrease was observed during the following 60 days.

Cell titer 96™ nonradioactivity cell proliferation assay (MTS assay)

Different concentrations of osteoblast cells were seeded on a 96 well Elisa plate to determine the cell viability and construct a calibration curve (Fig. 7). This calibration curve was used in the determination of the cell concentration inside the matrices and by this way, it was possible to quantitate the cells seeded inside the matrices.

Seeded materials

Osteoblast growth on PHBV foams with different pore sizes

The results showed that cell numbers proliferated more on large pore size foams (made by leaching out 300–500 μm sucrose crystals) than on small pore size foams (75–300 μm sucrose crystals) (Table I).

During seeding, cell concentration of 1×10^6 cells/mL seemed to be the ideal loading condition because cell growth was observed more on the high density cell suspension loaded foams (1×10^6 cells/mL) than that of the lower density cell suspension (1×10^5 and 5×10^5 cells/mL) loaded ones.

T A B L E I MTS assay for osteoblast growth after 7 days of incubation on PHBV; with different pore sizes and surface treatment

Sucrose type and surface treaments	Cell number ($\times 10^3$) after 7 days of incubation		
	Initial 1×10^5	Initial 5×10^5	Initial 10×10^5
Free cells	600 ± 11	975 ± 15	1221 ± 22
6% UT, SSC	160 ± 6	646 ± 10	1102 ± 11
6% UT, LSC	189 ± 8	680 ± 5	1120 ± 12
6% OP, LSC	240 ± 8	727 ± 5	1248 ± 10

UT: untreated PHBV; SSC: small sucrose crystal (75–300 μm) loaded PHBV; LSC: large sucrose crystal (300–500 μm) loaded PHBV; OP: rf-oxygen plasma treated (100 W, 10 min) PHBV.

Osteoblast growth on PHBV foams with different surface treatments

The surfaces of the large sucrose loaded PHBV matrices were treated with rf-oxygen plasma (100 W, 10 min) and osteoblast growth was studied on PHBV matrices. The rf-plasma treatment positively influenced cell-seeding efficiency (increased 6–24%, observed with the lowest seedings) (Table I). Since oxygen plasma treatment makes the surface more hydrophilic, it probably provides a better microenvironment for osteoblast growth than untreated PHBV. Optimum seeding concentration also peaked at 1×10^6 cells/mL.

In order to study the effect of extent of oxygen plasma modification of the foams on osteoblast cell growth inside the PHBV matrices, foams were treated at different levels (50 W for 10 min; 50 W for 20 min; 100 W for 10 min; and 100 W for 20 min).

Results showed that (Table II) untreated and 50 W for 10 min oxygen plasma treated foams allowed lowest populations of osteoblasts within their structures. As the treatment extent increased the level of occupation of the foam structure increased peaking at 100 W for 10 min. For the free cells, it was found that cell number was much lower than observed under similar conditions. This makes the results obtained more valuable since some OD values are even higher than those of the free cells indicating that the matrices provided a very suitable environment for cell growth.

ALP activity

For the determination of the effect of the cell-substrate interaction on the production of alkaline phosphatase, cells were cultured for 7, 14, 21 and 29 days on a polymer scaffold. ALP activity (expressed as $\mu mol\,min^{-1}\,cell^{-1}$) increased over time for all samples (Fig. 8). At all time points, the ALP activities of oxygen plasma treated (100 W 10 min) PHBV (0.59, 1.2, 3.6 and 4.8 $\mu mol\,min^{-1}\,cell^{-1}$, respectively) were higher than that of untreated PHBV (0.5, 1.0, 3.4 and 3.9 $\mu mol\,min^{-1}\,cell^{-1}$, respectively.

T A B L E I I Osteoblast growth after 7 days of incubation on PHBV foams with different oxygen plasma treatments as determined with MTS assay

Sample type	OD_{490}	Cell number
Free cells	1.620	$700\,000 \pm 10\,500$
Untreated PHBV	0.690	$300\,000 \pm 9300$
PHBV – 50 W, 10 min	0.693	$300\,000 \pm 5000$
PHBV – 50 W, 20 min	0.806	$350\,000 \pm 8500$
PHBV – 100 W 10 min	0.919	$400\,000 \pm 10\,000$
PHBV – 100 W 20 min	0.852	$370\,000 \pm 8100$

Discussion

PHBV matrices have a significant role in tissue engineering applications and the development of living tissue products for therapeutic applications [13]. They are reported to be biodegradable materials suitable for use in the construction of many biomedical products such as heart valves [14], blood vessels, pericardial substitutes, orthopedic applications [15] and drug release systems [16]. Unlike many other degradable polymers, the properties of PHB's can be changed by various treatments [13]. It is possible to add bioactive factors, alter surface and mechanical properties, and change degradation rate. They also have the advantage of being free of chemicals used in synthetic processes and are readily available in the range of molecular weights [6]. In the present study, PHBV was chosen as a temporary substrate, cell carrier or scaffold for osteoblast growth.

Surface characteristics and the pore size of the matrices are very important parameters for osteoblast growth. Since osteoblasts cannot grow without cell-to-cell contact, the average sizes of the pores should be at least 3 times larger ($> 100\,\mu m$) than that of the cells so that a single cell can find a chance to establish contact with the others. SEM results showed that untreated and unloaded PHBV had compact structure and a smooth surface leading to relatively less and small pores within the matrices. A solute (salt)-leaching technique was studied for the generation of porous foams [17,18]. In the present study, the size of the pores increased after sucrose leaching and became more suitable for cell growth. Ishaug-Riley *et al.* [19] showed that bone-like tissues can be created *in vitro* on PLGA scaffolds with pore sizes in the range of 150–710 μm. They observed that pore size and scaffold thickness did not affect the *in vitro* culture results such as proliferation, ALPase activity, and mineralization. However, it was demonstrated in the present study that scaffold pore size had an effect on osteoblast proliferation. Osteoblasts proliferated more on large pore size PHBV foams (300–500 μm) than on small pore size ones (75–300 μm).

Surface properties of the matrices are important since the surfaceis in direct contact with the cells [13]. Surface treatments, such as rf-oxygen plasma treatment, lead to creation of a more hydrophilic structure in comparison to the untreated material.

The rate and mode of degradation of the polymers influence their service life, mechanical properties and the

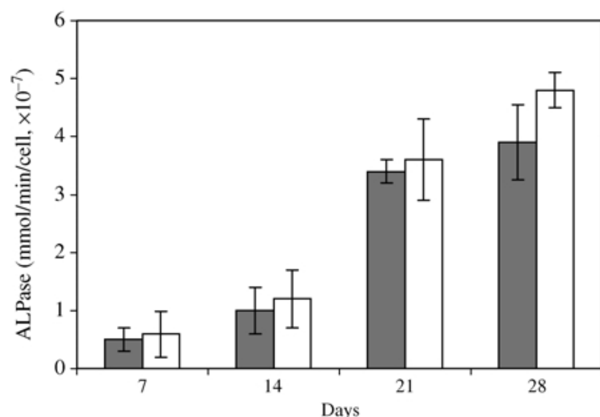

Figure 8 ALP activity of osteoblasts cultured inside the PHBV (6%, w/w) foams for 7, 14, 21 and 29 days. (■) untreated PHBV; (□) rf-oxygen plasma treated (100 W, 10 min) PHBV.

response of the biological system towards them. Bergsma *et al*. [20] reported that the degradation of polymerized PLLA takes 5.6 years for total resorption in the biological system. On the other hand, amorphous PLLA degrades in at most 1 year. Incorporation of glycolide into the polymer to create PLGA affects the degradation rate. Enhanced degradation rate can be obtained by increase in glycolide content. Also with the use of copolymers, the molecular weight decrease started after 2 weeks [21]. PHBV matrices are also degraded both *in vitro* and *in vivo* [22–25]. Holland *et al*. [24] studied *in vitro* degradation of PHBV20 in pH 7.4 buffer at 37 °C. In this study, weight remained almost unchanged for about 400 days. In another study, Yasin *et al*. [25] showed that the rate of weight loss from injection molded plaques of PHBV was increased by blending of sodium alginate, dextrin, amylose and talk. The present degradation study demonstrated the pH and weight changes for the unloaded and sucrose loaded 6% samples during 180 days. At the end of the incubation period, change in the pH was found to be insignificant in both samples since aging study was carried out in dilute buffer solution. Sample weights stayed the same in the first 120 days and then a 2-fold decrease was observed during the following 60 days. It appears that the samples absorb the solvent (water) and strain by swelling until 120 days and then chain scission starts shortening the chains and causing the changes in weight. There may also be chain scission in this first period but the shortened chains do not leave the structure before a critical chain length and extent of degradation is reached. These data indicate that the microenvironment of the loaded cells would become less restrictive as time progresses (at least after the initial 3 months) and, therefore, increases in the cell numbers within the foam structure would not be excessively hindered by space limitation.

After cell seeding onto the foams, MTS assay was carried out to determine the cell density inside the matrices. It was found that large size sucrose (300–500 μm) loaded PHBV (6%, w/w) foams treated with 100 W, 10 min were the optimal scaffold materials. During seeding, cell concentration of 1×10^6 cells/mL seemed to be the ideal loading condition. Osteoblast growth inside the matrices was determined by ALP. It proved the presence of osteoblasts inside the matrices. At the end of 21 and 29 days of incubation oxygen plasma treated (100 W, 10 min) foams showed higher amount of ALP activity than that of untreated PHBV.

It thus appears that tissue engineering of bone using oxygen plasma treated, large pore size PHBV matrices has a serious potential and current studies are concentrated on this group of materials.

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References

1. P. X. MA, R. ZHANG, G. XIAO and R. FRANCESCHI, *J. Biomed. Mater. Res.* **54** (2001) 284.
2. B. L. SEAL, T. C. OTERO and A. PANITCH, *Mater. Sci. Eng. R.* **34** (2001) 147.
3. J. M. POLLOK and J. P. VACANTI, *Seminars in Pediatric Surgery* **5** (1996) 191.
4. S. L. ISHAUG, G. M. CRANE, M. J. MILLER, A. W. YASKO, M. J. YASZEMSKI and A. G. MIKOS, *J. Biomed. Mater. Res.* **36** (1997) 17.
5. C. W. POUTON and S. AKHTAR, *Adv. Drug. Del. Rev.* **18** (1996) 133.
6. F. KOOSHA, R. H. MULLER and S. S. DAVIS, *Cri. Rev. Therap. Drug Carr. Sys* **6** (1989) 117.
7. E. FUKADA and Y. ANDO, *Int. J. Biol. Macromol.* **8** (1986) 361.
8. C. H. RIVARD, C. CHAPUT, S. RHALMI and A. SELMANI, *Ann. Chir.* **50** (1996) 651.
9. H. M. MULLER and D. SEEBACH, *Angew. Chem. Int. Engl.* **32** (1993) 477.
10. S. GOGOLEWSKI, M. JOVANOVIC, S. M. PERREN, J. G. DILLON and M. K. HUGHES, *J. Biomed. Mater. Res.* **27** (1993) 1135.
11. I. GÜRSEL, F. KORKUSUZ, F. TÜRESIN, N. G. ALAEDDINOĞLU and V. HASIRCI, *Biomaterials* **22** (2001) 73.
12. J. C. KNOWLES and G. W. HASTINGS, *Biomaterials* **12** (1991) 210.
13. S. F. WILLIAMS, D. P. MARTIN, D. M. HOROWITZ and O. P. PEOPLES, *Int. J. Biol. Macromol.* **25** (1999) 111.
14. R. SODIAN, HOERSTRUP, J. S. SPERLING, D. P. MARTIN, S. DAEBRITZ, J. E. MAYER and J. P. VACANTI, *J.P. ASAIO J.* **46** (2000) 107.
15. M. F. YAĞMURLU, F. KORKUSUZ, İ. GÜRSEL, P. KORKUSUZ, Ü. ÖRS and V. HASIRCI, *J. Biomed. Mater. Res.* **46** (1999) 494.
16. A. L. IORDANSKII, E. V. DIMITRIEV, P. P. KAMAEV and G. E. ZAIKOV, *J. Appl. Polym. Sci.* **74** (2000) 595.
17. P. X. MA and R. LANGER, in ''Tissue Engineering'', edited by M. Yarmush, J. Morgan (Humana Press Inc. Totowa, NJ, 1998).
18. A. G. MIKOS, A. J. THORSEN, L. A. CZERWONKA, Y. BAO, R. LANGER, D. N. WINSLOW and J. P. VACANTI, *Polymer* **35** (1994) 1068.
19. S. L. ISHAUG-RILEY, G. M. CRANE-KRUGER, M. J. YASZEMSKI and A. G. MIKOS, *Biomaterials* **19** (1998) 1405.
20. J. E. BERGSMA, F. R. ROZEMA, R. R. M. BOS, G. BOERING, W. J. BRUYJN and A. J. PENNINGS, *ibid.* **16** (1995) 267.
21. S. MING LI, H. GARREAU and M. VERT, *J. Mat. Sci. Med.* **1** (1990) 131.
22. I. GÜRSEL and V. HASIRCI, *J. Microencapsulation* **12** (1995) 185.
23. I. GÜRSEL, C. BALÇIK, Y. ARICA, O. AKKUS, N. AKKAS and V. HASIRCI, *Biomaterials* **19** (1998) 1137.
24. S. J. HOLLAND, M. YASIN and B. TIGHE, *ibid.* **11** (1990) 206.
25. M. YASIN, S. J. HOLLAND and B. J. TIGHE, *ibid.* **11** (1990) 451.

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