Influence of Hydroxyvalerate Composition of Polyhydroxy Butyrate Valerate (PHBV) Copolymer on Bone Cell Viability and *In Vitro* Degradation

Hui Liu,[1] M. Pancholi,[1] J. Stubbs III,[2] D. Raghavan[1]

[1]*Polymer Program, Department of Chemistry, Howard University, Washington, District of Columbia 20059*
[2]*Department of Microbiology, College of Medicine, Howard University, Washington, District of Columbia 20059*

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ABSTRACT: The objective of this study was to elucidate the role of hydroxyvalerate (HV) composition in polyhydroxy butyrate valerate (PHBV) copolymer film on the degradation of copolymer and osteoblastic cell activity. Degradation was studied by monitoring time-dependent changes in mass and chemical composition of the macroporous films. The mass loss of PHBV film upon 19 weeks of exposure to pH 7.4 phosphate buffer medium was found to range from 2.8% to 9.2% with a strong dependence on the original composition of the copolyester film and morphology. Tapping mode atomic force microscopy (TMAFM) was used to examine the roughness change of polyester films due to exposure to buffer medium. Chemical analysis of the degraded film was carried out using NMR to aid in the interpretation of the mass loss and TMAFM data. The NMR results showed a significant decrease in the mol % of HV content in the degraded PHBV film. Additionally, we established that UMR-106 cell proliferation on macroporous PHBV matrix is minimally enhanced by the HV content of PHBV copolymer. Information provided by this study can be used in the selection of appropriate PHBV copolymer for clinical use where the biopolymer needs to remain physically intact and chemically unchanged during the intended period of biomedical application. J Appl Polym Sci 116: 3225–3231, 2010

Key words: degradation; characterization; osteoblast cell viability

INTRODUCTION

There has been considerable scientific and technological interest in designing polymer devices for use in sutures, suture fasteners, staples, screws, bone plates, bone plating systems, surgical mesh, bone graft substitutes, guided tissue repair/regeneration devices, and bone marrow scaffolds.[1,2] Several natural and synthetic biopolymers have been studied but it is not clear which polymer is best suited for these applications. Although devices of polylactic acid (PLA), polyglycolic acid (PGA), and their copolymers have shown promising clinical results, design of surgical sutures and meshes from these polymers have often encountered several problems.[3] Some of these problems include the induction of the inflammatory response, poor cell growth on the biopolymer, and incomplete healing.[4]

Efforts to address these problems have focused in part on consideration of other biodegradable polymer(s) that support the growth of bone cells and promote the functions of the implanted device. Among the various biodegradable polymers explored poly(hydroxyalkonates) (PHAs) naturally produced by various microorganisms has drawn considerable interest for use in bone tissue engineering applications.[2,5–7] Of the various PHAs, only poly hydroxybutyrate (PHB), poly(hydroxybutyrate-*co*-hydroxyvalerate) (PHBV), poly(hydroxybutyrate-*co*-hydroxyhexanoate) (PHBHx), and polyhydroxyoctonate (PHO) can be produced in sufficient quantity for biomedical applications. Of the various PHAs abundantly available, PHB and PHBV are the most extensively studied polymers as biomaterial[8,9] under *in vitro* and *in vivo* conditions. Doyle et al.[10] demonstrated that devices based on PHB supported favorable bone tissue response without evidence of chronic inflammation during implantation periods of 12 months. However, one of the serious drawbacks of PHB is its brittle characteristic. Physical blending of PHB with other natural or synthetic polymers or synthesis of PHB copolymers by consideration of appropriate carbon source during fermentation has

Correspondence to: D. Raghavan (draghavan@howard.edu).
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led to improvements in mechanical and biological properties and successful fabrication of end product.[11] In addition, PHBV copolymers possess desirable characteristics such as low immunogenicity and nontoxicity to living tissues that are commonly sought in bulk material used for devices.[12]

PHBV copolymers has been shown to support favorable fibroblast cell growth at rates similar to that noticed in collagen sponges.[13–15] Recently, we reported an enhancement of osteoblast cell adhesion and growth on PHBV matrix modified with collagen (physical or chemical immobilization).[16,17] The cell attachment is believed to involve the adhesion receptors on the cell surface and proteins adsorbed to the PHBV surface that contain the collagen RGD (Arg-Gly-Asp) cell-binding domain sequence. The collagen-coated surface can initially facilitate cell anchoring to the PHBV matrix by providing cell-binding sites, however, with time collagen is washed from the physically immobilized surface by the culture medium, exposing the underlying PHBV film to support cell growth. As expected, chemically grafted collagen on PHBV film provided the most favorable matrix for cell proliferation followed by physically immobilized collagen compared with the unmodified film.[16,17] Additionally, we noticed that macroporous PHBV film supported nearly six times more cell proliferation than the nonporous scaffold.[18]

Long term degradability of the scaffold is one among the many widely used criteria in the selection of biodegradable polymer for medical applications. Most of the degradation studies reported in the open literature have centered on nonporous PHBV film. PHBV has been shown to degrade under a variety of environments, including aerobic or anaerobic conditions or composting medium.[19–21] Several reports describe degradability of PHBV in enzymatic (e.g., extracellular PHB depolymerase) and/or hydrolytic environments.[22–27] These studies have indicated that degradation of PHBV depends on several factors such as chemical composition, physical state of the polymer (granule, solvent cast film, and melt cast film), time, temperature, and the conditions of degradation. Since a number of these factors interplay in the degradation mechanism, understanding the degradation behavior is a complex issue.[20] At present, the information on the extent of hydrolytic degradation of PHBV film with various mol % of HV units in the copolymer is limited. At best, there are conflicting reports describing how the role of HV content of PHBV film impacts the hydrolytic degradation of PHBV.[22,24,27] A systematic study of PHBV hydrolytic degradation with varying HV content is needed to develop a better understanding of the role of HV composition on PHBV degradation. Little is known about the role of the macroporous structure scaffold in influencing the overall degradability of PHBV film.

In this study, we investigate the degradation of macroporous and nonporous PHBV film in a phosphate buffer medium at 37°C, by preparing PHBV film with varying mol % of HV content ranging from 0 to 12%. The *in vitro* degradation of PHBV film was followed by examining the chemical changes of the PHBV film using NMR measurements and physical changes by recording mass loss of the film and film roughness by atomic force microscopy (AFM). Additionally, the effect of HV composition in PHBV copolymer osteoblast cell viability was investigated.

MATERIALS AND METHODS

Materials

All reagents were used as purchased from the supplier without further purification. Poly(hydroxybutyrate) (PHB), Poly(3-hydroxybutrate-*co*-3-hydroxyvalerate) containing 5 wt % hydroxyvalerate (PHBV5), Poly(3-hydroxybutrate-*co*-3-hydroxyvalerate) containing 8 wt % hydroxyvalerate (PHBV8), Poly(3-hydroxybutrate-*co*-3-hydroxyvalerate) containing 12 wt % hydroxyvalerate (PHBV12), sodium chloride, potassium bromide (FTIR grade), and d-chloroform (solvent for NMR analysis) were purchased from Sigma-Aldrich (St. Louis, MO). Chloroform was purchased from Fisher Scientific (Hampton, NH).

Preparation of macroporous and nonporous PHBV films

Solvent cast macroporous PHBV film was made by mixing 1.05 g of PHBV powder with 1.50 g of sieved sodium chloride (~ 150 μm) manually and dispersing it in 10.5 mL of chloroform at 60°C. The mixture was cast on a glass petri dish at room temperature. The cast solution was allowed to air dry at room temperature for about 24 h so as to allow solvents to evaporate. The solvent remnant in the film was removed by subjecting the film to vacuum drying for additional 24 h at room temperature. The PHBV/NaCl composite film was released from the petri dish and thoroughly washed in 500 mL of distilled water at room temperature for several days. The wash solution was replaced every 2 h for the first 8 h and then two to three times a day to remove the leachable component from the film. The solution was checked for the presence of chloride ions by testing the filtrate with 0.1*N* $AgNO_3$ for the formation of AgCl precipitate. The washing of the PHBV film was continued until the filtrate was free of chloride ions. The salt leached PHBV film was air and vacuum dried at room temperature. Drying process

was continued until the mass of the film was constant. The entire drying process lasted up to 2 weeks. Macroporous films of thickness (340 μm) and HV content of 0, 5, 8, and 12% were prepared using similar procedure. Additionally, we also prepared nonporous PHBV films by dissolving PHBV in chloroform (in the absence of salt particles) and film casting.

Degradation of PHBV film

PHBV films (initial mass ranging from 100 to 200 mg; initial film thickness, 340 μm) were placed in screw top bottles that contained 40 mL of phosphate (NaH_2PO_4 and Na_2HPO_4) buffer medium (adjusted to pH 7.4). The bottles were placed in a water bath, which was maintained at 37 ± 2°C. Specimens were periodically removed, washed with deionized water, and dried to constant mass in vacuum at ambient temperature. The dried films were saved for AFM and ^{1}H-NMR studies.

Analysis of degraded film

Gravimetric analysis

The mass loss of the macroporous and nonporous PHBV films upon exposure to buffer medium was determined gravimetrically. Percentage of mass loss is defined as a change in mass with respect to the initial mass and is expressed as follows:

$$\text{Mass loss}\,(\%) = \frac{M_o - M_t}{M_o} \times 100\% \qquad (1)$$

where M_o and M_t represent the initial mass and the mass of the PHBV film at time t in the buffer medium. The reported mass results are averages of a minimum of three sample measurements, unless otherwise stated.

Atomic force microscopy (AFM)

TMAFM was used to obtain the roughness of PHBV film before and after exposure to phosphate buffer medium. It should be mentioned that since the pores in the film were macroscale, AFM measurements were limited to nonporous regions of the porous PHBV film or nonporous PHBV film. A multimode atomic force microscope (AFM Digital Instruments, Dimension 3100, Veeco, Santa Barbara, CA) was operated in tapping mode under ambient conditions with commercial silicon probes (tip radius of 5 to 10 nm and spring constant from 20 to 40 N/m, Veeco, Santa Barbara, CA). The film was scanned and images of scan size (1 μm × 1 μm) were collected for the PHBV film. Mean roughness of unexposed and buffer medium exposed PHBV film was calculated from these images by using DI software.

^{1}H-NMR

High-resolution ^{1}H-NMR analysis of PHBV was performed on a Bruker Ultra Shield-400 MHz NMR spectrometer. The ^{1}H-NMR spectra were recorded at 27°C of $CDCl_3$ solutions of undegraded and degraded PHBV by acquiring 16 scans. Deutrated chloroform was used as both the solvent and the internal standard for NMR characterization of unexposed and buffer medium exposed PHBV film.

Osteoblast cell activity measurement on macroporous PHBV film

Details about the preparation of porous PHBV film for the cell viability study can be found in earlier publications.[18,28] Briefly, the porous film was treated with 2.2 g/h oxygen mixed ozone for 20 min at room temperature and collagen was immobilized by dip coating the ozone-treated film in a 4 mg/ml collagen solution (prepared in 0.3% acetic acid) for 24 h and then air drying.

12×10^4 cells were seeded on collagen physisorbed PHBV film, whereas 3.0×10^4 cells were seeded on TCPS matrix in 200-μl serum free DMEM media. The cells along with the test matrix were incubated at 37°C in 5% CO_2 for 20 h. After 20 h, nonadherent cells were removed via aspiration and PHBV scaffolds with adherent cells were transferred to a new 96 well that contained DMEM supplemented with 10% fetal bovine serum and incubated for extended time period. At predetermined time intervals (4d and 6d), the cells that proliferated on the PHBV film and on TCPS matrix were assayed by the MTS method for the metabolic activity.

MTS reagent was prepared by adding the novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) (10 μl) to 200 μl of phenazine methosulfate (PMS) and mixing with 840 μl of growth medium.[18,28] One hundred fifty microliters of the prepared reagent was added to the proliferated cells on the test matrix, and then incubated at 37°C for 2.5 h. An aliquot (125 μl) of the reacted reagent was recovered from the well and the absorbance of the solution was recorded by a Dynatech Laboratories' Microplate Reader 5000/7000 at 490 nm.

Treatment of data

The data are presented in the form of mean ± standard deviation (Mean ± SD). Three measurements ($n = 3$) were recorded for each set of samples, with the exception of few samples where two measurements

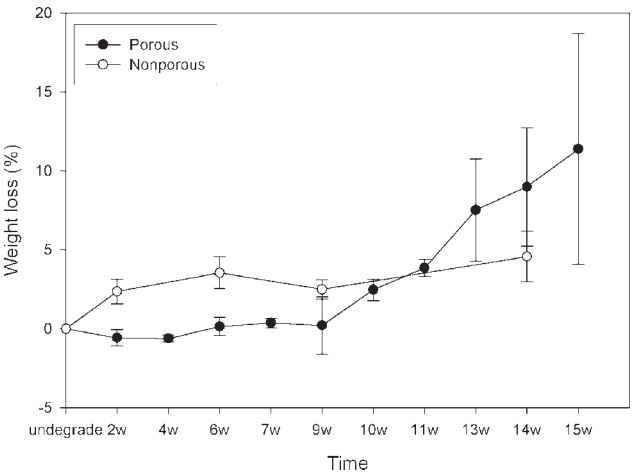

Figure 1 The mass loss of porous and nonporous PHBV films as a function of exposure time.

were reported. The Student's *t*-test was performed on the data collected from various PHBV films and the difference between measurements was considered statistically significant when $P < 0.05$. The statistical analysis of the data was performed using SigmaPlot statistical package software.

RESULTS AND DISCUSSIONS

In an earlier study, we showed that macroporous PHBV film could be prepared by initially solvent casting the salt/PHBV chloroform mixture and solute leaching of the cast film.[18,28] The morphology of leached film and pristine PHBV film was characterized by optical microscopy and atomic force microscopy.[18] Pores were noticed in the porous PHBV film and the characteristic dimension of the pores ranged from 45 μm to around 150 μm, which was consistent with the dimension of the salt particles used in the study. TGA characterization of the unleached and leached salt/PHBV composite films was performed to confirm that sodium chloride was indeed washed out of the leached salt/PHBV composite film to form macroporous PHBV film.[28]

Measurement of bulk degradation by mass loss analysis

The mass loss measurement provides vital information about bulk degradation of PHBV film with 8 mol % HV. Figure 1 shows the mass loss profile of porous and nonporous PHBV films as a function of degradation time over 15 weeks period. Beyond 15 weeks, the mass loss measurement was discontinued because hydrolytic degradation resulted in breaking of the porous film into several small pieces. The residual pieces could not be fully accounted for determination of mass loss of PHBV film.

For the porous sample, we noticed minimal mass loss during the initial 7 weeks and a more robust mass loss beyond the initial degradation period. Albertsson and coworkers[21] noticed a similar trend in hydrolytic degradation of PHBV film when exposed to sterile water at 60°C for 49 weeks. It seems that the mass loss is directly proportional to degradation time and film morphology. For macroporous PHBV film, we noticed nearly twice the mass loss as that of nonporous PHBV film. This is because 3D porous matrix with interconnected porosity provides pathway for degradation media to percolate the bulk film and promote degradation.[29,30]

Figure 2 shows the mass loss of various macroporous PHBV films with varying HV content as a function of degradation time. The degradation results showed that maximum mass loss occurs in PHBV film with 12 mol % HV content compared with PHBV film with 0 mol % HV content. After 19 weeks degradation, PHBV with 12 mol % HV showed a mass loss of ~ 9.2%, whereas PHBV with 8 mol % HV showed a mass loss of ~ 8.0 %, whereas PHBV with 5 mol % showed a mass loss of ~ 6.2% and PHB showed a mass loss of ~ 2.8%. It should be mentioned that the mass loss data reported for macroporous PHBV film with 8 mol % HV in Figures 1 and 2 slightly differ and this may have to do with the source of PHBV used in film formation. For Figure 1, PHBV film was casted from PHBV powder, whereas for Figure 2, the PHBV film was casted from PHBV strips. In general, the reported mass loss results are in agreement with Holland et al.[22] where HV content in the PHBV copolymer was shown to affect its *in vitro* degradation. Additionally, our results are in contrast to the observations reported by Doi et al.,[24] where solvent cast

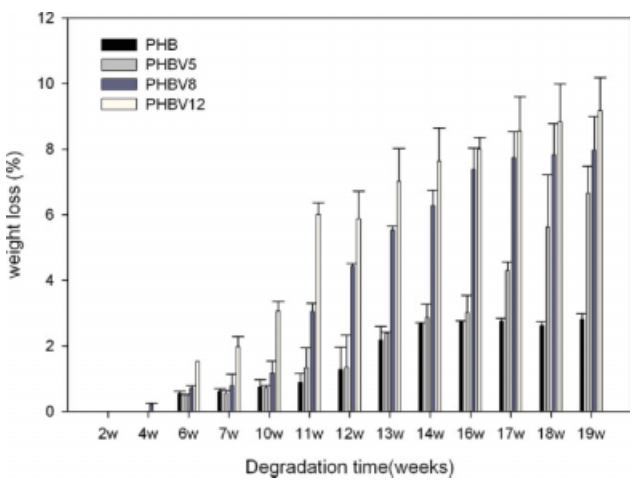

Figure 2 The mass loss of porous PHBV films with varying HV content as a function of degradation time. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

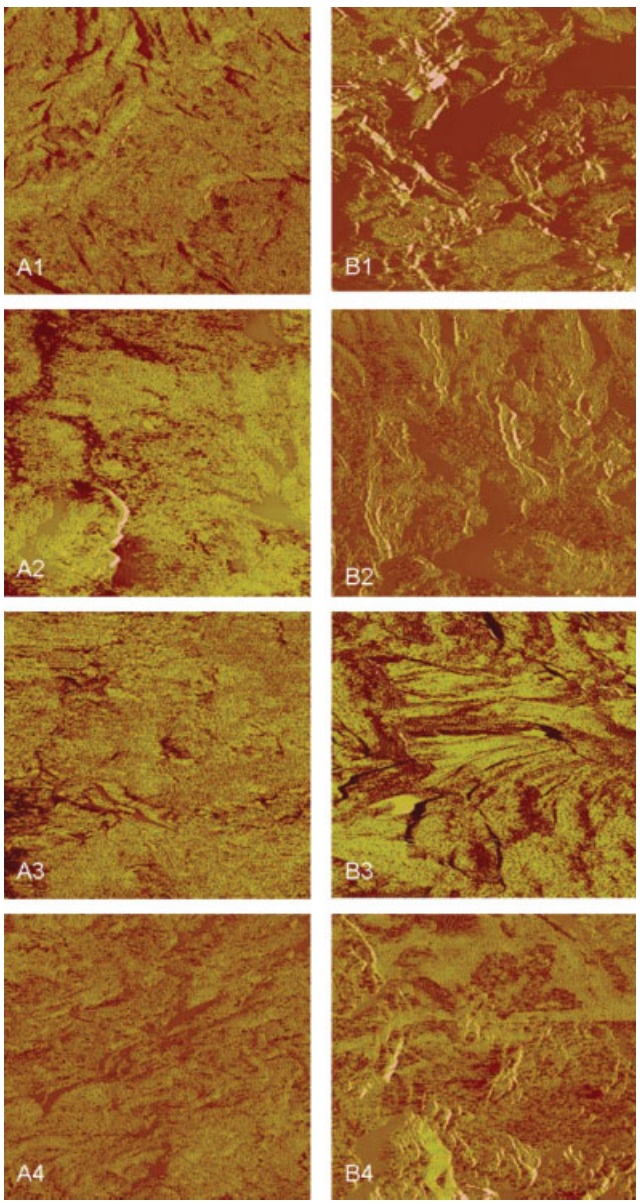

Figure 3 Atomic force microscopy images of porous PHBV films with scanning size 5 μm × 5 μm (A1) undegraded PHB; (B1) PHB immersed in phosphate buffer medium for 18 weeks; (A2) undegraded PHBV5; (B2) PHBV5 immersed in phosphate buffer medium for 18 weeks; (A3) undegraded PHBV8; (B3) PHBV8 immersed in phosphate buffer medium for 18 weeks; (A4) undegraded PHBV12; (B4) PHBV12 immersed in phosphate buffer medium for 18 weeks. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

films of PHBV with 45% HV and 71% HV showed no major mass loss over a period of 58 days. It must be mentioned that Holland et al.[22] and Doi et al.[24] performed hydrolytic degradation on PHBV film with significantly different HV content. Because the HV content of the investigated copolymer(s) in Holland et al.[22] and this study are relatively low, it is conceivable that the mo1 % of HV in the PHBV copolymer has a significant role to play in the observed mass loss. It appears that there may be a maxima or break point in the mass loss profile as suggested by Albertsson and coworkers,[21] beyond which increasing the mo1 % of HV in the PHBV copolymer does not necessarily influence the extent of PHBV degradation.

Characterization of surface degradation by atomic force microscopy

To investigate whether the morphology of PHBV film changes during degradation, the microstructure of the PHBV film before and after degradation was characterized by TMAFM. Figure 3 is the two-dimensional topographic images for undegraded and degraded PHBV film by TMAFM. A heterogeneous structure was observed on length scales less than 5 μm in the topographic images of degraded and undegraded PHBV film. Previous studies provide some evidence on polyester microstructure and its characteristic heterogeneity.[31] Several AFM mages of 1 μm × 1 μm scan dimension were collected and the root mean square roughness (RMS) of PHBV films was obtained. The RMS is defined as the standard deviation of height above an average plane. Table I summarizes the RMS data of undegraded and degraded PHBV film and it was found to increase for PHBV12 film from 7.4 nm to 15.1 nm after exposure to buffer medium which suggests that the degradation medium causes surface erosion of PHBV film. Unlike plasma-treated PHBV film in air or nitrogen where surface erosion was considerable, the surface erosion due to hydrolytic environment exposure was minimal.

To verify that the observed minimal surface roughness changes were indeed due to the exposure of PHBV film to hydrolytic environment, the images of several PHBV films with different HV content were collected by TMAFM technique. The AFM images of exposed film showed heterogeneous structure with marginal increase in surface roughness compared with unexposed film.

Chemical characterization of degraded and undegraded PHBV film

To relate the physical changes (mass loss and roughness increase) in hydrolytically degraded PHBV film

TABLE I
AFM Roughness Data (RMS) for Undegraded and Degraded PHBV

Type of PHBV film	Undegraded (nm)	Degraded (nm)
PHB	10.965	18.340
PHBV5	12.755	20.535
PHBV8	13.453	23.116
PHBV12	7.432	15.130

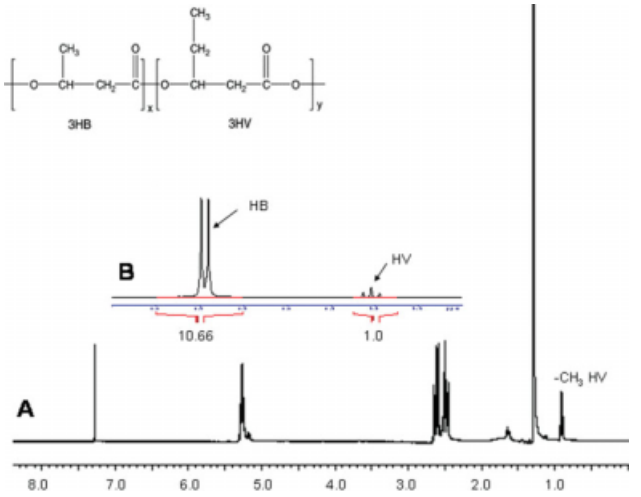

Figure 4 (a) ^{1}H-NMR spectrum of PHBV films; (b) the HB and HV methyl resonances were expanded from the same ^{1}H-NMR spectrum. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to changes in chemical structure, NMR spectra of several PHBV films before and after exposure to hydrolytic medium were collected. Figure 4 shows the typical ^{1}H-NMR spectrum of a PHBV sample containing 8 mol % HV. The characteristic peaks at 0.9 ppm and 1.25 ppm have been assigned to the resonance absorption of methyl (CH_3) from hydroxyvalerate unit and methyl (CH_3) from hydroxybutyrate unit, respectively. Our peak assignment was further verified with the ^{1}H-NMR spectrum of PHB film that showed only one characteristic peak at 1.25 ppm for the resonance absorption of methyl (CH_3) from hydroxybutyrate unit. Bloembergen et al.[32] has shown that the characteristic peaks at 0.9 ppm and 1.25 ppm in the NMR spectrum can be used to determine the HV composition in the PHBV sample according to the following equation:

$$\text{HV composition}\,(\%) = \frac{\text{Area}\,CH_3\,(\text{HV})}{\text{Area}\,CH_3\,(\text{HV}) + \text{Area}\,CH_3\,(\text{HB})} \times 100\% \quad (2)$$

By integrating the area under the peaks at 0.9 ppm and 1.25 ppm in the spectra of the individual PHBV sample and using eq. (2), it was found that PHBV5, PHBV8, and PHBV12 sample has an HV content of 6.1%, 9.9%, and 11.0%, respectively. These results are in general agreement with the HV content of the PHBV sample supplied by the manufacturer.

Figure 5 shows the HV content (as determined by ^{1}H-NMR) in PHBV5 film exposed to buffer medium for 19 weeks at 37°C. We followed the degradation of PHBV5 film as a function of exposure time and noticed a gradual decrease in the HV content over the 19-week degradation period. The drop in the HV content of degraded PHBV film mirrors the data reported by Albertsson and coworkers,[21] for hydrolytically degraded PHBV6 film at 60°C. The drop in HV content of the copolymer suggests that the HV regions in the copolymer are preferentially abiotically hydrolyzed.

To establish whether the HV content in the original copolymer is indeed influenced by the hydrolytic degradation of PHBV film, ^{1}H-NMR spectra of the PHBV5, PHBV8, and PHBV12 film after 19 weeks degradation in buffer medium was collected and HV content in the copolymer was determined. The HV content in the degraded film was found to be 5.4%, 8.0%, and 8.6%, for PHBV5, PHBV8, and PHBV12 film, respectively. We compared the HV content of degraded copolymer to the original copolymer composition and noticed a maximum decrease in the HV content of PHBV12 film (ΔHV = 2.4%) followed by PHBV8 film (ΔHV = 1.9%) and then PHBV5 film (ΔHV = 0.7%). This trend mirrors the mass loss data of degraded PHBV copolymer as a function of HV content in PHBV copolymer after 19 weeks exposure to buffer medium. Since maximum mass loss and the biggest drop in HV content of the copolymer was noticed for the degraded PHBV12 sample, these results again indicate that the HV units are the sites of hydrolytic degradation. Water may act as a plasticizer and enter the amorphous HV regions of PHBV to cleave the ester bonds leading to chain scission of polyester units.[30–32]

Effect of HV composition of porous PHBV films on cell viability

We have selected several PHBV films with varying HV composition (e.g., PHB, PHBV5, PHBV8, and PHBV12) to investigate the effect of HV composition in PHBV copolymer on cell viability. 12×10^4 UMR-106 cells were seeded on collagen coated, 20-min

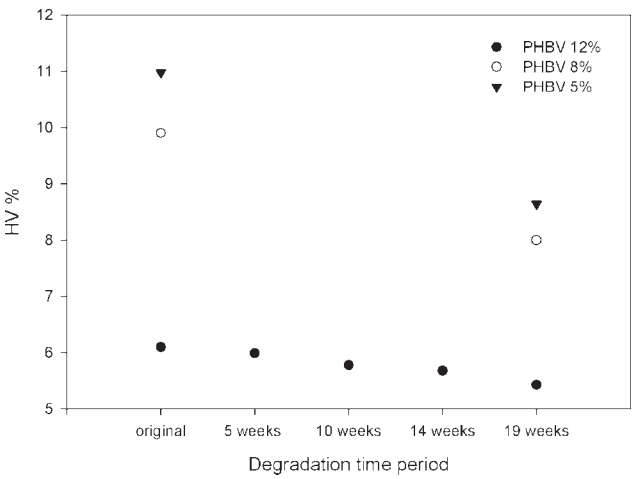

Figure 5 HV composition of the PHBV films after degradation in buffer medium at 37°C for 19 weeks, as determined from ^{1}H-NMR measurement.

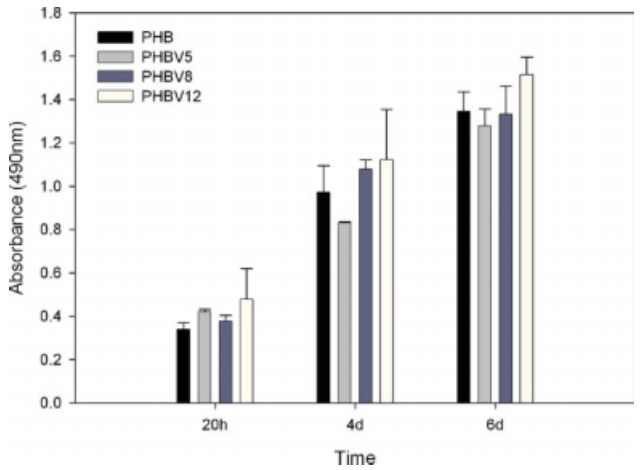

Figure 6 A plot of relative cell proliferation on porous PHBV scaffolds as a function of HV composition. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ozone-treated porous PHB, PHBV5, PHBV8, and PHBV12 film, respectively. Figure 6 compares the extent of cell attachment and cell proliferation after 20 h, 4d, and 6d on the various PHBV matrices. The results show that the cell attachment and proliferation on the various PHBV matrices are at best marginally influenced by the HV content of the PHBV film. We also conducted cell attachment and cell proliferation measurements on TCPS (data available) and noticed a greater number of cells attached to TCPS but far fewer cells proliferated on the PHBV matrix indicating limited nutrients available for cells to grow. Further studies are needed to verify this observation.

CONCLUSIONS

In this article, we investigated the *in vitro* degradation of PHBV with varying HV composition in phosphate buffer medium. Mass loss results show that the mol % of HV in the PHBV copolymer influences the extent to which PHBV degrades in phosphate buffer medium. We noticed slower degradation of PHBV film for up to 7 weeks beyond which there was a steady degradation of PHBV film. The HV content in the copolymer was found to decrease upon extended exposure to hydrolytic medium. The biggest drop in HV content of the copolymer was noticed for PHBV12 film, as ascertained by ^{1}H-NMR measurements. The initial slow degradation of PHBV followed by its steady degradation at pH 7 and 37°C suggests that PHBV-based biomaterials may remain intact for several weeks before degrading in physiological environments, Moreover, this degradation property makes PHBV an ideal material for orthopedic surgical implants that require stability for a short period of time. Additionally, the biocompatibility study showed marginal enhancement in osteoblast-like cell attachment and proliferation. This increase in cell growth is due to the increase in the HV copolymer content of PHBV film and/or the collagen presence in the PHBV matrix. The ability of osteoblast cells to proliferate on the PHBV matrix and the initial stability of the matrix are useful characteristics which makes PHBV a suitable candidate for orthopedic applications.

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