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# Biocompatibility of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) Copolyesters Produced by *Alcaligenes* sp. MT-16

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**Abstract** Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), poly(3HB-*co*-3HV), copolyesters, with 3-hydroxyvalerate (3HV) contents ranging from 17 to 60 mol%, were produced by *Alcaligenes* sp. MT-16, and their biocompatibility evaluated by the growth of Chinese hamster ovary (CHO) cells and the adsorption of blood proteins and platelets onto their film surfaces. The number of CHO cells that adhered to and grew on these films was higher with increasing 3HV content. In contrast, the tendency for blood proteins and platelets to adhere to the copolyester surfaces significantly decreased with increasing 3HV content. Examination of the surface morphology using atomic force microscopy revealed that the surface roughness was an important factor in determining the biocompatibility of theses copolyesters. The results obtained in this study suggest that poly(3HB-*co*-3HV) copolyesters, with >30 mol% 3HV, may be useful in biocompatible biomedical applications.

*Keywords*: biocompatibility, blood compatibility, cell compatibility, polyhydroxyalkanoates, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)

## INTRODUCTION

Polyhydroxyalkanoates (PHAs), polyesters of hydroxyalkanoic acids, are synthesized by a wide range of bacteria, usually under unbalanced growth conditions, in order to act as carbon and energy reserve materials [1]. PHAs are promising materials for various applications, because they have useful mechanical properties, as well as being biodegradable and biocompatible. Therefore, PHAs have recently attracted interest in biomedical and pharmaceutical fields, such as for tissue engineering and as drug delivery systems [2,3].

Polyhydroxybutyrate (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), poly(3HB-*co*-3HV), copolyesters are part of a large family of PHAs. In particular, poly (3HB-*co*-3HV) is of great commercial interest, since it exhibits a considerable range of mechanical properties, depending on the 3-hydroxyvalerate (3HV) content [4,5]. The production of poly(3HB-*co*-3HV) is usually achieved by providing bacteria with *co*-substrates, such as propionic acid and valeric acid, along with a main carbon source. Although the 3HV fraction in poly(3HB-*co*-3HV) increases as the concentration of the *co*-substrate in the culture medium increases, the maximum 3HV content in poly(3HB-*co*-3HV) is limited (generally less than 20 mol%) by the toxic effect of the *co*-substrates at relatively low concentrations [6]. Poly(3HB-*co*-3HV) copolyesters with high 3HV content are not necessarily more useful, but the ability to alter the 3HV content in poly(3HB-*co*-3HV) is desirable from an industrial viewpoint, as this offers the possibility of producing a range of different thermoplastics with varying degrees of flexibility and toughness.

The biocompatibility of PHB and poly(3HB-*co*-3HV) has been studied by a large number of research groups [7-10] and has been found to have low toxicity, in part due to the fact that it degrades *in vivo* to D-3-hydroxybutyrate, a normal constituent of human blood [7]. Although inherently biocompatible and biodegradable, the use of PHB is significantly limited in biomedical applications due to its shortcomings, *i.e.* its rigidity, brittleness and low mechanical properties. The poly(3HB-*co*-3HV) copolyesters are less crystalline and more flexible than PHB [5]. Therefore, their mechanical properties make them suitable for biomedical applications. The biocompatibility of poly(3HB-*co*-3HV) copolyesters has been investigated intensively, but most of the available data are for copolyesters containing less than 25 mol% 3HV [9]. Reports on the biocompatibility of poly(3HB-*co*-3HV) with high molar fractions of 3HV are relatively rare because of the difficulty in the preparation of such polymers. Therefore, the effect of the 3HV content on the biocompatibility of the poly(3HB-*co*-3HV) copolyesters remains

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to be well documented.

*Alcaligenes* sp. MT-16 is a threonine overproducing mutant, which accumulates poly(3HB-*co*-3HV), with significantly high 3HV content, when glucose is used as the sole carbon source [11]. Furthermore, the molar fractions of 3HV in the copolyester can increase greatly up to 77 mol%, when the organism is cultivated in a glucose medium supplemented with a *co*-substrate, such as levulinic acid. In a previous study, poly(3HB-*co*-3HV) copolyesters were prepared with varying 3HV contents, ranging from 17 to 60 mol%, by growing the organism in glucose medium supplemented with various concentrations of levulinic acid, and their degradability characteristics and thermo-mechanical properties were reported to be greatly influenced by the 3HV content of the copolyesters [12]. The aim of this study was to elucidate both the blood- and cell compatibilities of solution-cast films of poly(3HB-*co*-3HV) copolyesters. The biocompatibilities of the copolyesters were evaluated by the growth of Chinese hamster ovary (CHO) cells and the adsorption of blood proteins and platelets onto the surface of copolyester films. PHB and poly(L-lactide) (PLLA) were used as control materials to evaluate the biocompatibilities of the poly (3HB- *co*-3HV) copolyesters prepared during this study.

## MATERIALS AND METHODS

### Materials

The PHB was produced by *Wautersia* (formerly *Ralstonia*) *eutropha* KHB-8862, as previously described [13]. Poly(3HB-*co*-3HV) copolyesters with different 3HV contents were produced by growing *Alcaligenes* sp. MT-16 on a basal medium containing 20 g/L of glucose and levulinic acid in various ratios [11]. After 24 h of batch cultivation in a 5 L jar fermenter, the cells were harvested by centrifugation, followed by lyophilization. The polyesters were extracted from the lyophilized cells, using hot chloroform in a Soxhlet apparatus, and purified by precipitation with methanol [14,15]. A series of four poly (3HB-*co*-3HV) copolyester specimens, with 3HV contents of 17, 23, 36, and 60 mol%, were prepared, and labeled P(3HB-17-3HV), P(3HB-23-3HV), P(3HB-36-3HV) and P(3HB-60-3HV), respectively. The thermo-mechanical properties of these copolyesters, as well as their degradability characteristics, have been reported previously [12]. PLLA ($M_w$=110,000, Boehringer Ingelheim, Ingelheim, Germany) was used as purchased. All other chemicals and solvents were of analytical grade, and were also used with no further purification.

The PHB, poly(3HB-*co*-3HV) and PLLA films were prepared by dissolving the purified polymers in chloroform and casting the film on glass plates. The chloroform was evaporated by allowing the glass plate to stand at room temperature to reach equilibrium crystallinity.

### Blood Compatibility Test

Human albumin and fibrinogen were used to study the adsorption behaviours of the proteins onto the surfaces of the PHB, poly(3HB-*co*-3HV) copolyesters and PLLA. All blood proteins were purchased from Sigma. Small disks (15 mm in diameter) of polymer films were prepared using a punch and immersed in protein solutions, containing 1 mg/mL Dulbecco's phosphate buffered saline (PBS, pH 7.4; Sigma, St. Louis, MO, USA), at 37°C for 1 h. The disks were then recovered, and the changes in the protein concentrations of the solution borne proteins determined using a UV-spectrophotometer.

Platelet-rich plasma (PRP) was prepared by collecting human blood in plastic syringes containing 3.8% sodium citrate PBS solution to prevent coagulation. The mixture was centrifuged at 1,300 rpm for 10 min at 4°C, and the supernatant collected. Polymer disks, washed with PBS for 24 h, were placed in the bottom of the wells of a multiwell tissue culture plate, and the PBS solution removed from the multiwell tissue culture plate by pipetting. PRP (1 mL) was then seeded, incubated at 37°C for 30 min, the disks recovered and then rinsed three times with PBS to remove any weakly adsorbed platelets. After being fixed in 2.5% (v/v) glutaraldehyde solution, the morphology of the adsorbed platelets was observed using a scanning electron microscope (SEM).

### Cell Compatibility Test

CHO cells (CHO-K1, Korean Collection for Type Cultures) were used to study the effect of the 3HV contents on the cell compatibility of poly(3HB-*co*-3HV), which in turn affects the growth behavior of cultured cells. Polymer disks, washed with PBS for 24 h, were placed at the bottom of the wells of a multiwell tissue culture plate, and the PBS solution removed from the multiwell tissue culture plate by pipetting. The cells ($4 \times 10^4$ cm$^{-2}$) were then seeded to the polymer surfaces. Ham's F-12 nutrient mixture (Gibco Laboratories, USA), containing 5% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL gentamycin was used as the culture medium. The cells were cultured in an incubator at 37°C under a 5% $CO_2$ atmosphere. At the end of each incubation period, the supernatant was withdrawn and each well, washed with PBS, trypsinized (0.05% trypsin/0.02% ethylene-diamine-tetraacetic acid, Gibco), and the detached cells counted using a hemocytometer. The morphology of the cultured cells, fixed in 2.5% glutaraldehyde solution, was observed using a SEM.

### Analytical Procedures

The PHA content and composition were determined by gas chromatography, as described elsewhere [16], using poly(3HB-*co*-3HV) standards with known 3HB and 3HV monomer contents. SEM images were obtained using a Philips XL 30S scanning electron microscope. The samples were sputter-coated with platinum (150 Å) on a model Polaron SC7610 sputter coater. SEM measurements were obtained at 3 kV, with the secondary electron images recorded on a Polaroid film. The surface roughness of the film samples was examined using a

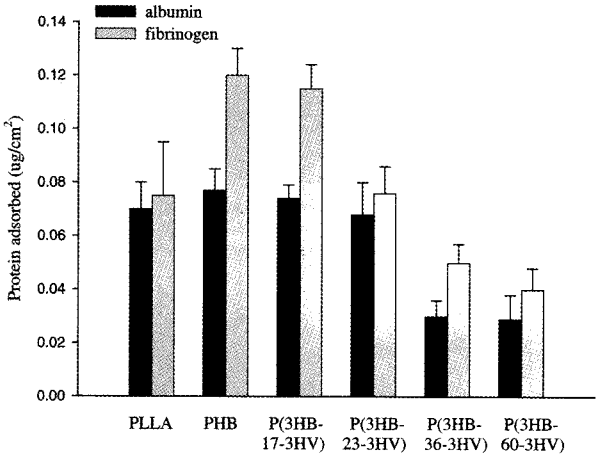


**Fig. 1.** Adsorption of proteins on the PLLA, PHB and poly (3HB-*co*-3HV) copolyesters. Error bars indicate the range of experimental readings obtained (sample number, $n$ = 3).

Nanoscope III atomic force microscope (AFM), in the tapping mode (scan size 80 μm, set point 3.3 V, scan rate 0.4 Hz). The mean square roughness ($R_a$) of the sample surface was evaluated directly from the AFM image.

## RESULTS AND DISCUSSION

### Blood Compatibility

Adverse behaviour of blood components at the interface with a foreign material is an essential problem in the biomedical applications of polymers [17]. The exposure of blood to artificial surfaces often causes the adsorption of blood proteins, which results in blood coagulation. Thus, obtaining detailed knowledge about protein adsorption onto foreign surfaces can help to improve the anti-thrombogenicity of the materials used in blood-contacting devices. It is generally recognized that preadsorbed albumin surfaces are passive, while preadsorbed fibrinogen surfaces activate platelet deposition [18-20]. In addition, platelet adhesion onto a surface is invariably followed by the appearance of platelet aggregates, platelet spreading, and subsequent thrombus formation, which are the potential dangers associated with the use of artificial materials *in vivo*. Blood compatibility is reached when the interaction between platelets and the material is minimized. Thus, the smaller the number of platelets at the material surface, the more blood compatible the material [21].

The results of the measurement of the protein adsorption of albumin and fibrinogen onto the prepared samples are shown in Fig. 1. The amounts of proteins adsorbed onto the surfaces of PLLA and PHB were larger than those onto the surface of the poly(3HB-*co*-3HV) copolyesters. Moreover, the amounts of protein adsorbed onto the poly(3HB-*co*-3HV) copolyesters decreased with increasing 3HV content. The interactions of the platelets with the surfaces of the PLLA, PHB and poly(3HB-*co*-3HV) copolyesters were investigated using PRP prepared from human whole blood. The surface morphology of the platelets adhered onto the polymer films are shown in Fig. 2. Fig. 2 shows that the generation of pseudopods from the platelets adhering on the polymer films increased in the order of PLLA, PHB and poly(3HB-*co*-3HV) copolyesters. For the poly(3HB-*co*-3HV) copolyesters films, the platelets were deformed less with increasing 3HV content. More specifically, the tendency for platelets to adhere was highly suppressed when the 3HV content was 60 mol%. These results clearly showed that both the adsorption of proteins and adhesion of platelets were suppressed by the increased 3HV content in the copolyesters. The protein adsorption and platelet adhesion to polymer surfaces are

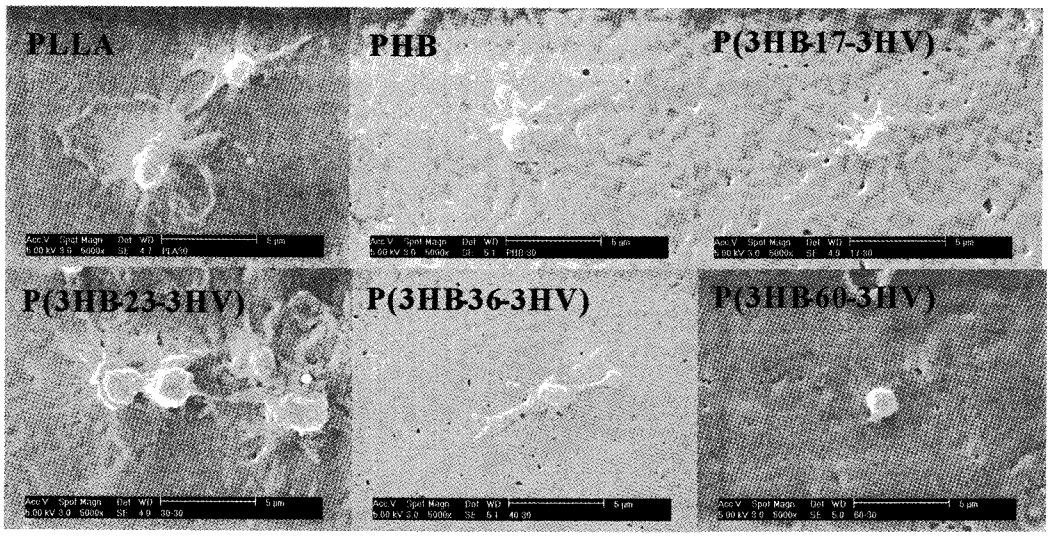


**Fig. 2.** Images of platelets adhered to the surfaces of the PLLA, PHB and poly(3HB-*co*-3HV) copolyesters, as observed by SEM (original magnification, 5,000 ×; current, 60 pA; spot size, 3.0 nm; vacuum, $10^{-5}$ mbar).

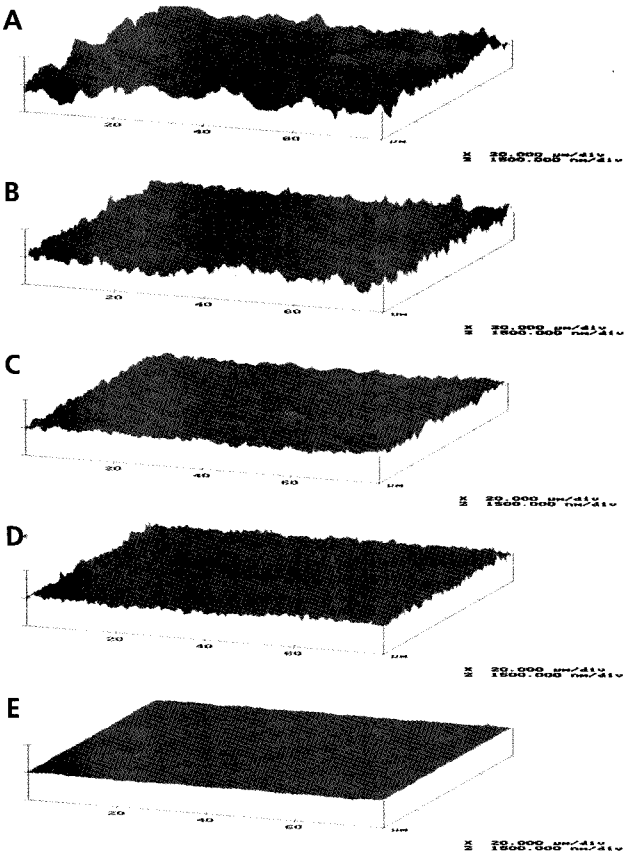


**Fig. 3.** Images of PHB (A), P(3HB-17-3HV) (B), P(3HB-23-3HV) (C), P(3HB-36-3HV) (D), and P(3HB-60-3HV) (E), as observed by AFM.

influenced by various factors, including the surface compositions, wettability, surface charges and roughness [22-24]. Hydrophilic surfaces, such as hydrogels, and PEG-grafted surfaces have been reported to suppress protein adsorption, as well as platelet adhesion, in a number of studies [25,26]. The surfaces of poly(3HB-*co*-3HV) copolyesters become more hydrophobic with increasing 3HV content [12]. However, the results of the present study showed that the most hydrophilic PLLA and PHB had more protein adsorption and platelet adhesion than the poly(3HB-*co*-3HV) copolyesters.

Since surface roughness is one of the factors affecting the protein adsorption and platelet adhesion to polymer surfaces [24], the surface roughness of the poly(3HB-*co*-3HV) copolyesters was examined by AFM. Fig. 3 shows the three-dimensional AFM images of the PHB and poly(3HB-*co*-3HV) copolyester surfaces. The mean square surface roughness ($R_a$) in an 80×80 μm surface region for PHB was about 860 nm. In contrast, the poly(3HB-*co*-3HV) copolyester surfaces became more regular and smooth with increasing 3HV content. The $R_a$ in an 80 × 80 μm surface region of the 60 mol% 3HV film decreased to about 16 nm. The observed differences in the initial surface roughness between the PHA samples might have been due to the lower degree of crystallization

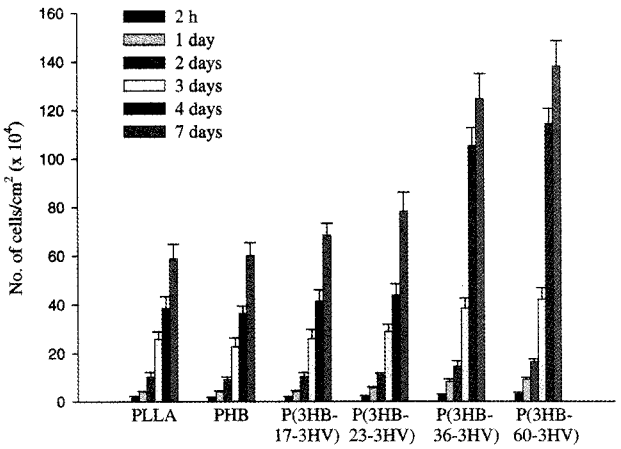


**Fig. 4.** CHO cell adhesion and growth on the surfaces of the PLLA, PHB and poly(3HB-*co*-3HV) copolyesters (number of seed cells, 4 × $10^4$ cm$^{-2}$). Error bars represent standard deviations from the means of eight samples.

and slower crystallization rate of the copolyesters with increasing 3HV content. The present results suggest that the blood compatibility of the poly(3HB-*co*-3HV) copolyesters is more dependent on the surface roughness than on their hydrophilicity.

## Cell Compatibility

CHO cells are popularly used as a model system because they can exist as reasonably stable single cells and are not unreasonably fastidious in terms of culture requirement [27]. The CHO cells were cultured for up to 7 days on the PLLA, PHB and poly(3HB-*co*-3HV) copolyesters. The culture medium was changed every 24 h. Fig. 4 shows that the number of cells grown on the sample films increased with increasing culture time. The growth rates of the cells on the poly(3HB-*co*-3HV) copolyesters were faster than those on the PLLA and PHB. For the poly(3HB-*co*-3HV) copolyester films, the growth of cells increased dramatically with increasing 3HV content. Cells cultured on the copolyesters with >30 mol% 3HV showed higher rates of proliferation and, therefore, a higher cell density compared to those obtained on the PLLA and PHB films. As mentioned above (Fig. 3), the surface morphology of the poly(3HB-*co*-3HV) copolyesters was smooth and the surface smoothness increased with increases in the 3HV content. These results indicate that the poly(3HB-*co*-3HV) copolyesters with >30 mol% 3HV provided a more regular and smooth surface for the cells to attach to and grow on thus, presented a better cell compatibility than PLLA and PHB. A similar result was reported by Washburn *et al.* [28], who investigated the sensitivity of osteoblastic MC3T3-E1 cells on PLLA films using a gradient in annealing temperature, which resulted in changes to nanometer-scale structure. They indicated that smoother surfaces also led to better cell adhesion, spreading and growth of osteoblasts.

Fig. 5 shows the SEM images of cells adhering to dif-

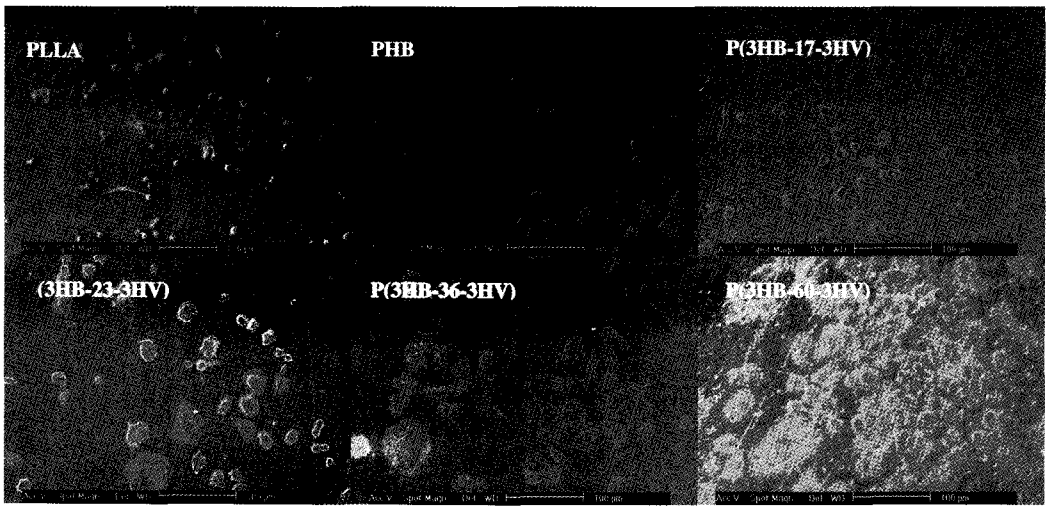


**Fig. 5.** SEM images of CHO cells, cultured for 24 h, on the surfaces of the PLLA, PHB and poly(3HB-*co*-3HV) copolyesters (original magnification, 200 ×; current, 60 pA; spot size, 3.0 nm; vacuum, $10^{-5}$ mbar).

ferent surfaces that were cultured for 1 day. The morphology of the cells adhered to the poly(3HB-*co*-3HV) films was significantly different from those of the PLLA and PHB. The cells on the poly(3HB-*co*-3HV) films were more spread than those on the PLLA and PHB. In particular, the generation of pseudopods from the cells adhering to the poly(3HB-*co*-3HV) copolyesters with 60 mol% 3HV was the most spread and flattened, suggesting again that the surface roughness of the PHA films was an important factor in determining cell attachment and proliferation.

## CONCLUSIONS

The results of the AFM measurements showed that the surfaces of the poly(3HB-*co*-3HV) films became more regular and smooth with increasing 3HV content. The results obtained from the protein and platelet adhesions, and cell growth tests showed that the biocompatibility of the poly(3HB-*co*-3HV) copolyesters were more improved with increasing 3HV content. The results obtained in this study suggest that poly(3HB-*co*-3HV) copolyesters, with >30 mol% of 3HV, have the potential, due to their excellent blood- and cell compatibilities, for use in a broad range of biomedical applications.

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