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Fabrication of Biodegradable Polymer Nanocomposite from Copolymer Synthesized by *C. necator* for Bone Tissue Engineering

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Abstract: poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV) composites were fabricated via the incorporation of nano-sized hydroxyapatite (nano-HA) using a solution casting method.Surface characteristics, pore distribution on PHBV surface were studied by scanning electron microscopy (SEM). pH change caused by degradation indicated the degradation extent and therefore, the sample that leads to the largest pH drop is the one that has degraded the most. But quick drop in pH indicated rapid degradation for PHBV/HA sample.

Key words: PHBV/HA · Hydroxyapatite (nano-HA) · *Cupriavidus necator* DSMZ 545 · Degradation

INTRODUCTION

Polyhydroxyalkanoates (PHA) are a class of polyesters that are synthesized and accumulated as an intracellular carbon and energy storage material by many bacteria. Poly(hydroxybutyrate-cohydroxyvalerate) (PHBV), a member of the PHA, has attracted the attention of academia and industry because of its natural origin, biodegradability, biocompatibility and thermoplasticity [1]. Nevertheless, it is not fully competitive compared to conventional polymer because there are some drawbacks to its further application. PHBV has a high melting temperature with highly crystalline solids and large spherulitic structures with slow growth. Because of its high crystallinity, PHBV is stiff and brittle, which results in very poor mechanical properties [1, 2]. Moreover, PHBV is unstable at a temperature near its melting temperature, which results in a drastic reduction in molecular weight during the melt processing [3]. Some attempts have been made to enhance the thermal stability and the physical and mechanical properties of PHBV, including blending or filling techniques [4, 5]. The incorporation of nanoparticles has provided the means to improve material performance with low filler contents.

The copolymers of hydroxybutyrate with hydroxyvaleric acid are less crystalline, more flexible and more readily processable than PHB itself [6, 7]. Their various properties such as natural origin, biodegradability [8], biocompatibility [5, 9], streospecificity, piezoelectricity [10], optical activity and thermoplasticity make them suitable for a variety of applications in health industry [5]. The biocompatibility of PHB and PHBV has been studied by a number of different research groups [11]. PHB 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 [12].

PHBV is now being studied intensely as a tissue engineering substrate. Rivard *et al*. [13] demonstrated that PHBV (9%) sustained a fibroblast proliferation rate similar to that observed in collagen sponges for at least 35 days. In addition, the PHBV materials maintained their integrity during the culture period while the collagen foams contracted substantially. Moreover, the total protein production after 4 weeks in culture was found to be twice as high in the PHBV foam as in the collagen foam. It thus showed that porous PHBV materials could be more than adequate as polymeric substrates for cell cultures. Hydroxyapatite (HA), a mineral quite similar in composition to the inorganic component of bone, is used to modify the mechanical properties of polymeric implants for certain medical applications. Composites of PHBV and HA with partial biodegradability and high mechanical strength and osteoconductivity were reported to be suitable for fracture fixation [14]. A composite of PHB reinforced with HA particles was tested as a bone analog and new bone growth at the interface of implantation site was observed after 6 months [15]. It thus appears to be suitable filler for improving mechanical properties and bone healing. Medical devices Synthetic biodegradable polymers have attracted considerable attention for applications in medical devices and will play an important role in the design and function of medical devices.

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The general criteria of polymer materials used for medical devices include mechanical properties and a degradation time appropriate to the medical purpose. In addition, the materials should not evoke toxic or immune responses and they should be metabolized in the body after fulfilling their tasks. According to these requirements, various synthesized biodegradable polymers have been designed and used. Some synthesized biodegradable polymers that have been used or show potential in selected fields are summarized below [6].

Biological polymers degrade rapidly in the biological medium when appropriate hydrolytic enzymes are available. On the other hand, since synthetic organic polymers are produced via chemical synthesis, they disappear slowly from the environment [16]. The rate and mode of degradation of the polymers influence their service life, mechanical properties and the response of the biological system towards them.Bergsma *et al*. [17] reported that polymerized PLLA is totally resorbed in 5.6 years in the biological system. On the other hand, amorphous PLLA degrades in at most 1year. Incorporation of glycolide into the chain (PLGA) enhances the degradation rate. Also with the use of copolymers, a decrease in the molecular weight started after 2 weeks [18]. PHBV matrices are also degraded both *in vitro* and *in vivo* [19-22] but PHB and PHBV are generally degraded slower than corresponding PLA and PLGA. Holland *et al*. [21] studied *in vitro* degradation of PHBV20 in pH 7.4 buffer at 371C in which the weight of the sample remained almost unchanged for about 400 days. These long degradation durations could be shortened by various treatments and also by blending. For example, Yasin *et al*. [22] showed that the rate of weight loss from injection molded plaques of PHBV was increased by blending with sodium alginate, dextrin, amylose and talcum powder.

In this study, a nanocomposite based on bioresorbable polymer poly(3-hydroxybutyrate-co-3-hydroxyval) (PHBHV) was prepared by the incorporation of nano-sized hydroxyapatite (nano-HA) using a solution casting method. Also the degradation of this kind of nanocomposites in compare of biopolymers has been discussed.

MATERIALS AND METHODS

Preparation of PHBHV Copolymers: A mineral salt medium which consisted of: 1.0 g/l $(NH_4)Cl$, 2.3 g/l KH_2PO_4, 2.9 g/l K_2HPO_4, 1 g/l $MgSO_4.7H_2O$, 10 mg/l $CaCl_2$, 50 mg/l, Fe (NH_4), 0.5 g/l $NaHCO_3$, 5 ml/l trace metal solution, 0.5 g/l yeast extract and 1.0 g/l peptone. The trace metal solution consisted of: 2.2 g/l $MgSO_4.7H_2O$, 0.1 g/l $FeSO_4.5H_2O$, 0.08 g/l Zn $SO_4.7H_2O$, 2.2 g/l K_2SO_4, 0.02 g/l H_3BO_3, 0.08 g/l $CuSO_4$. Glucose was used as carbon source with concentration of 40 g/l for preparation of seed culture and media used as inoculums. Glucose, yeast extracts and peptone, K_2HPO_4, Fe (NH_4), $NaHCO_3$ and trace metal solution were sterilized separately at 121°C and then aseptically reconstituted at room temperature prior to inoculation. The media used for comparison studies with variation of carbon and nitrogen sources concentration has the same composition except the described variable term varied. With concentration variation of $(NH_4)Cl$ and glucose as nitrogen and carbon sources media were prepared for these sets of experiments.

Molasses was obtained from sugar industry (Shirvan, Ian). Whole molasses solution was uniformly acidified by acid solution (Hcl, 5N) at acidic pH (less than 4) to remove excessive proteins [23]. The supernatant was refrigerated for 12 hours and it was used after adjusted pH to 7 by the concentrated NaOH solution (5M), as the major constituent of media for the growth of *C. necator* in all experiments. In the present research, acetate was used as a supplementary carbon source. The biosynthesis of PHA copolyesters containing 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) units from molasses and acetate were investigated [23].

Preparation of PHBHV/HA Nanocomposite: PHBHV solution in chloroform were prepared by fermentation of molasses and acetate by *C.necator* and followed by addition of a certain amount of commercially available hydroxyapatite (HA) nanoparticles with a primary crystal size of 20-30 nm. After 10 min ultrasonication (25 kHz, 200 W) in) in a water bath at 50°C, the resulting mixtures were vigorously stirred again at the same temperature for 3 h. Subsequently, the well-mixed PHBHV/HA solution was poured into a Petri dish and the nanocomposite film was obtained after being dried at 60°C for more than 12 h for fabrication of testing samples [23].

FTIR Spectroscopy: Infrared spectra (IR) were recorded on 1 mg biopolymer films cast from chloroform solution onto 300 mg KBr (Merck) plates by using FTIR (Shimadzu, FTIR 1650) at 27 °C.

RESULTS AND DISCUSSION

The IR spectrum of standard PHBV and produced sample are shown in figure 1 and figure 2.There were two strong adsorption band at 1250 cm^{-1} and 1750 cm^{-1} which are characteristic for ester bonding. Other adsorption bands at 3000 and 3400 cm^{-1} for -CH and O-H groups respectively are given in Figure 2.

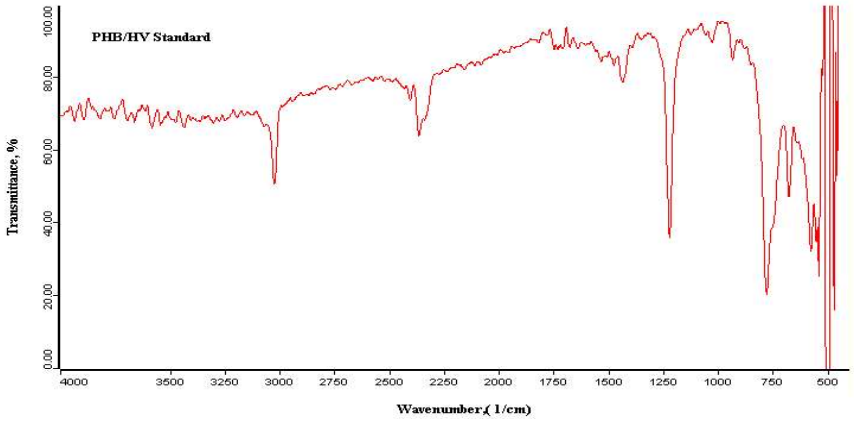

Fig. 1: IR Spectrum of the standard PHB/HV comopolymer

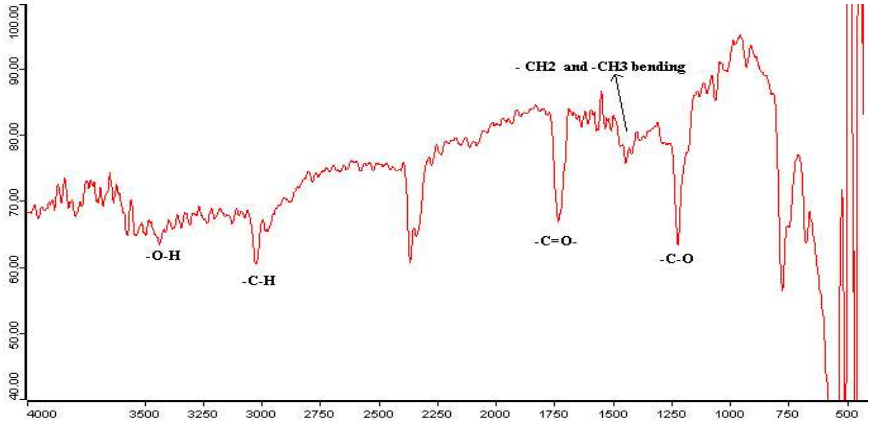

Fig. 2: IR Spectrum of the produced PHB/HV comopolymer

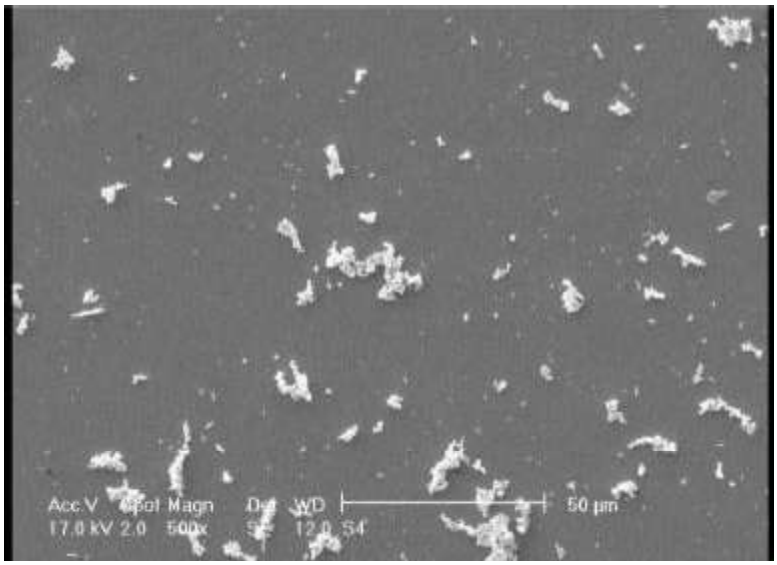

Fig. 3: SEM micrograph of poly(3-hydroxybutyrate-co-3-hydroxyval) (PHBV)

In this research, in order to improve the dispersion of nanoparticles in the polymer matrix (Figure 3), a solution casting method combined with a strong ultrasonication was introduced. The SEM (Philips, model XL30, Netherlands) examinations revealed that HA particles have been well dispersed and evenly distributed in

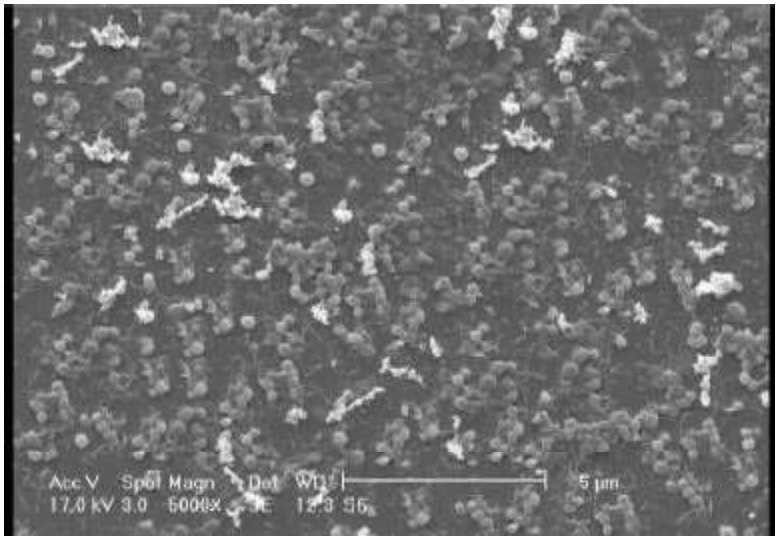

Fig. 4: SEM micrographs of poly(3-hydroxybutyrate-co-3-hydroxyval)/ hydroxyapatite PHBV/HA nanocomposite

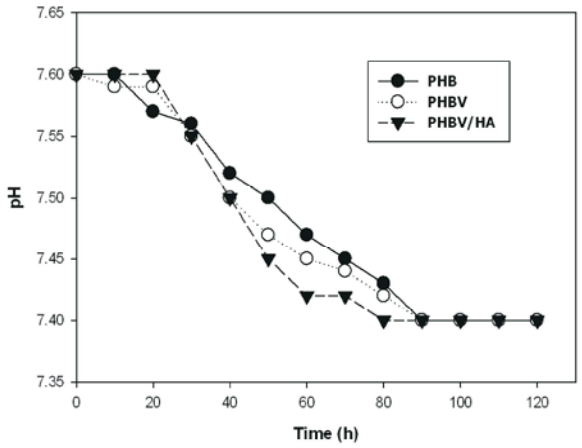

Fig. 5: Change in the pH of the medium as an indication of degradation of PHB, PHBV and PHBV/HA samples

the polymer matrix (Figure 4). No clear evidence of agglomeration can be found in the Macromolecular nanocomposites. The good dispersion of inorganic fillers in the nanocomposites benefits the improvement of mechanical properties of composite materials.

Degradation of PHBV/HA: Biological polymers degrade rapidly in the biological medium when appropriate hydrolytic enzymes are available. On the other hand, since synthetic organic polymers are produced via chemical synthesis, they disappear slowly from the environment [24]. The rate and mode of degradation of the polymers influence their service life, mechanical properties and the response of the biological system towards them. Bergsma *et al*. [25] reported that polymerized PLLA is totally resorbed in 5.6 years in the biological system. On the other hand, amorphous PLLA degrades in at most 1 year. Incorporation of glycolide into the chain (PLGA) enhances the degradation rate. Also with the use of copolymers, a decrease in the molecular weight started after 2 weeks [26]. PHBV matrices are also degraded both *in vitro* and *in vivo* [27-30] but PHB and PHBV are generally degraded slower than corresponding PLA and PLGA. Holland *et al*. [29] studied *in vitro* degradation of PHBV20 in pH 7.4 buffer at 371C in which the weight of the sample remained almost unchanged for about 400 days. These long degradation durations could be shortened by various treatments and also by blending. For example, Yasin *et al*. [30] showed that the rate of weight loss from injection molded plaques of PHBV was increased by blending with sodium alginate, dextrin, amylose and talcum powder. In the degradation experiment, PHB, PHBV and PHBV/HA samples were used. Changes in pH of the aqueous incubation media with time were determined. PHB led, in 120 days, to a pH decrease of 0.18 pH units and a decrease of 0.19 pH units were observed with PHBV sample. Meanwhile PHBV/HA led to pH decreases of 0.19 units in120 days. This pH decrease is an indication of the degradation extent and therefore, the sample that leads to the largest pH drop is the one that has degraded the most (Figure 5).

But quick drop in pH indicated rapid degradation for PHBV/HA sample.

CONCLUSION

PHBV was reported to be biodegradable material suitable for use in the construction of heart valves,blood

vessels, pericardial substitutes, orthopedic biomaterials and in drug release systems [31]. In the present study, PHBV was tested as a temporary substrate for nanocomposite fabrication. SEM used to study the surface characteristics, pore distribution of PHBV matrices showed that the surface and the bulk of the carrier were strikingly similar in this regard. pH change caused by degradation indicated the degradation extent and therefore, the sample that leads to the largest pH drop is the one that has degraded the most. But quick drop in pH indicated rapid degradation for PHBV/HA sample.

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