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Nanocomposite (PHBHV/HA) Fabrication from Biodegradable Polymer

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Abstract: Biopolymer composite materials for potential medical applications are of current research interest. 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. To improve the dispersion of nanoparticles in the polymer matrix, a strong ultrasonication was introduced. Homogeneous distribution of nanoparticles in the polymer matrix was validated by the observation of scanning electron microscope (SEM).

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

INTRODUCTION

Poly (3-hydroxybutyrate) (PHB) is fully biodegradable, thermoplastic aliphatic polyester with biocompatibility and ecological safety, produced in nature by at least 75 different genera of bacteria as an energy storage material [1]. Its physical properties are often compared to isotactic polypropylene since they have similar melting points and crystallinity [2]. However, it has several drawbacks, such as stiffness, brittleness and very low thermal stability at processing temperatures that prevent its larger commercial applications. The thermal instability of PHB in the melt prevents it from substituting the non-biodegradable polymeric materials in commercial products [3]. That is why improvement of the thermal stability of PHB is very important. To overcome these drawbacks of PHB we have prepared PHB nanocomposites with organically modified montmorillonite [4].

Polymer nanocomposites are commonly defined as the combination of a polymeric matrix and fillers that have at least one dimension (i.e. length, width or thickness) in the nanometer size range [5]. It has been shown that only a few percent of nanofillers (usually from 1 to 5 wt.%) leads to greatly improved thermal, mechanical and barrier properties of polymers [6-8]. Of this kind, however, polyhydroxyalkanoate (PHA) /hydroxyapatite nanocomposites are seldom concerned to the best of our knowledge. Hydroxyapatite (HA) - ($Ca10(PO_4)_6(OH)_2$) has been widely used as bioactive material for its chemical similarity to the major inorganic component of bone. Because of its excellent biocompatibility and osteoconductivity, HA has been used as a drug carrier in various drug delivery systems, especially in bone tissue treatment [8].

In this study, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV), one of the most well-known PHA, was applied as the matrix. The PHBHV/HA nanocomposite was prepared by using solution casting method. Doyle *et al.* [9] demonstrated that materials based on PHB produce a consistent favorable bone tissue adaptation response with no evidence of an undesirable chronic inflammatory response after implantation periods up to 12 months. Bone is rapidly formed close to the material and subsequently becomes highly organized, with up to 80% of the implant surface lying in direct apposition to new bone. The materials showed no conclusive evidence of extensive structural breakdown in vivo during the implantation period of the study. Particulate hydroxyapatite (HA) incorporated into PHB forms a bioactive and biodegradable composite for applications in hard tissue replacement and regeneration.

In addition, PHB/HA and PHBV/HA composites have a mechanical strength in compression of 62MPa, which is about the same order of magnitude of several human bones and thus it is interesting as biomaterial for use in fracture fixation [10]. In vivo study using HA/PHBV bone-implants to be implanted into the tibias of rabbits was found to be morphologically, biologically and chemically active throughout the period of study. There was a strong tendency to rebuild the bone structure at the interface after implantation. In regions about

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50-100 mm from the interface, the bone region displayed an osteon organization. Osteoblasts and osteocytes were identified throughout the interface region. The thickness of the newly formed bone significantly increased over the period of the experiment from about 130 mm at 1 month to about 770 mm at 6 months [11].

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.

MATERIALS AND METHODS

Microorganism: The microorganism used in the present study was *Cupriavidus necator* DSMZ 545 (Deutsche Sammlung von Mikroorganismen und Zellkulturen) for culture propagation. The stock culture was stored and maintained on Luria Agar slants at 4°C. The organism was sub-cultured every 15 days to maintain its viability.

Media: 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 and 0.08 g/l $CuSO_4$. Molasses was used as carbon source preparation of seed culture and media used as inoculums. Molasses, 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.

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 [12, 13]. 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.

Biopolymer Extraction: For biopolymer extraction, a considerable bacteria from fermentation process after 48 hr were centrifuged, then dried in 50°C Oven for 24hr. Dried cells mixed with chloroform (1:50) and then the mixture was put in Soxhlet apparatus in 60°C during 16 hr for destroying cell wall. After that extracted biopolymer was solved in chloroform. For excluding destroyed cell wall, the solution was filtered by 0.45 micron filter. Clear solution after filtering was added to cold methanol methanol/Water water solution (70:30) while it was shaking for extraction of biopolymer. A milky solution has been obtained. After the solution was stabled and the phases were separated, the lower phase was evaporated in room temperature to remove methanol. For more purity, we can put the biopolymer obtained from first stage into Soxhlet apparatus with chloroform again and the rest stages were repeated respectively [13].

NMR Spectrometry: ^{13}CNMR spectra of biopolymer samples and fractions were collected on a Brukera Bruker, DRX 400, Germany spectrometer in the pulse Fourier Transform mode. The 400 MHz ^{1}H NMR spectra were recorded at 30°C using 5×10^{-3} g mL^{-1} g/mL PHBV solutions in $CDCl_3$ (6.0 s pulse repetition;8 kHz spectral width; 80 scans). The 150 MHz ^{13}C proton-noise decoupled NMR spectra were recordedatrecorded at 30°C using 25×10^{-3} g mL^{-1} PHBV solutions in $CDCl_3$ (5.0 s pulse repetition; 30 kHz spectral width;15,000 scans). Tetramethylsilane (Me_4Si, $\delta = 0$) was used as an internal chemical shift standard.

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 Commerciallycommercially 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.

RESULT AND DISCUSSION

The sequence distribution of 3-hydroxyvalerate (HV) and 3-hydroxybutyrate (HB) comonomers in PHBV copolyesters and their fractions was determined from ^{13}CNMR spectra (Figure 1).

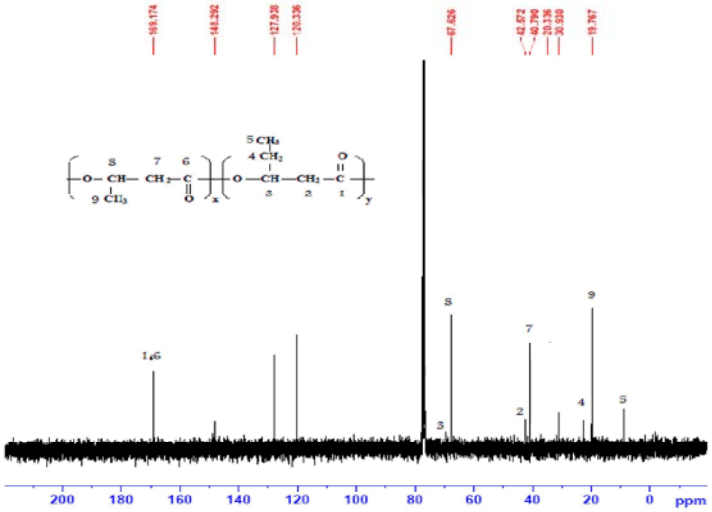

Fig. 1: The ^{13}C NMR spectra of PHBV copolyesters

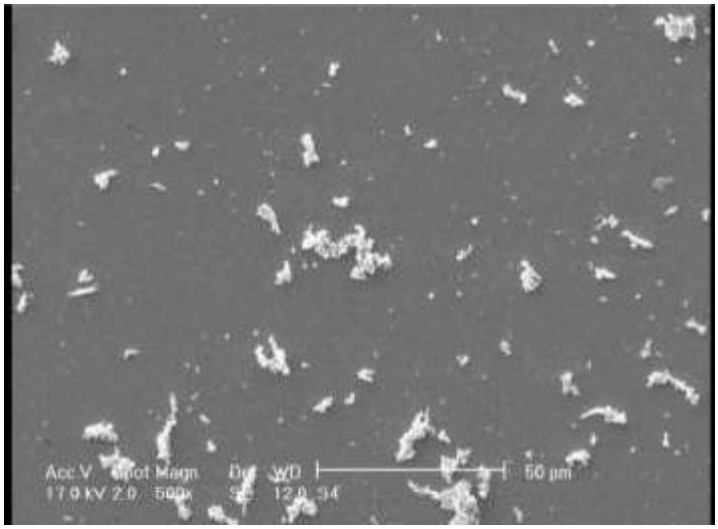

Fig. 2: SEM micrograph of poly(3-hydroxybutyrate-co-3-hydroxyval) (PHBV)

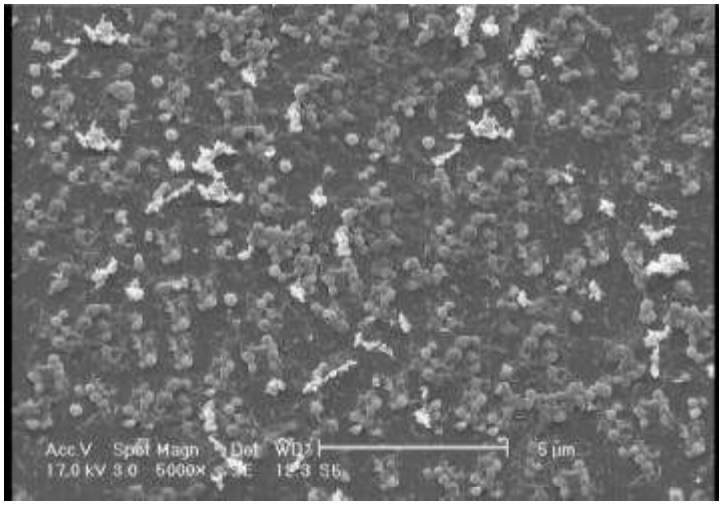

Fig. 3: SEM micrographs of poly(3-hydroxybutyrate-co-3-hydroxyval)/ hydroxyapatite PHBHV/HA nanocomposite

The ^{13}C NMR ($CDCl_3$, ppm) shown in ascertained the chemical composition of the copolymer. The peaks at 9 ppm, 19.9 ppm (CH_3), 23.6, 23.6 ppm, 40.8 ppm,42.6, 42.6 ppm (CH2), 67.6 ppm, 67.9 ppm (CH), 169.2 ppm (CO) belonged to PHB blocks and the peak at 70.7 ppm was characteristic of main chain methylene units in the PEG blocks.

Ultrasonication stirring has been proven to be an effective strategy to overcome the agglomeration of particles in the biopolymer [14]. In this research, in order to improve the dispersion of nanoparticles in the polymer matrix, a solution casting method combined with a s trong ultrasonication was introduced. The SEM) Philips, model XL30, Nitherland) examinations revealedexaminations revealed that HA particles haveparticles have been well dispersed and evenly distributed in the polymer matrix (Figure 2). No clear evidence of agglomeration can be found in the nanocomposites (Figure 3).

The good dispersion of inorganic fillers in the nanocomposite, inevitably benefits the improvement of mechanical properties of composite materials [4]. Finally In this work, the PHBHV/HA nanocomposite has been successfully fabricated by using solution casting method with an aid of strong ultrasonication.

CONCLUSIONS

The combination of osteoconductive properties of hydroxyapatite with the processability of biodegradable polymer overcomes limitations induced by the brittleness and can widen the application potential in bone tissue engineering. In this work, the PHBHV/HA nanocomposite has been successfully fabricated by using solution casting method with an aid of strong ultrasonication. Due to the enhanced bioactivity and mechanical properties in comparison with conventional composite, the PHBHV/HA nanocomposite supports a more promising potential for the repair and replacement of bone.

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