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Biocompatibility of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) with bone marrow mesenchymal stem cells

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Abstract

As a new member of the polyhydroxyalkanoate (PHA) family, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) (PHBVHHx) was produced by recombinant *Aeromonas hydrophila* 4AK4. PHBVHHx showed a rougher surface and had higher hydrophobicity than the well-studied polymers poly(L-lactic acid) (PLA) and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx). Human bone marrow mesenchymal stem cells (MSCs) adhered better on PHBVHHx film than on tissue culture plates (TCPs), PLA film and PHBHHx film. The cell number on the PHBVHHx film was 115% higher than that on the TCPs, 66% higher than on the PHBHHx film and 263% higher than on the PLA film ($p<0.01$). PHBVHHx also supported the osteogenic differentiation of MSCs. Previous studies have shown that all PHA polymers tested were either poorer than or equal to TCPs for supporting cell growth. PHBVHHx is the only PHA polymer to significantly increase cell numbers compared with TCPs. These data demonstrate that PHBVHHx could be a promising biomaterial for bone tissue engineering.

Keywords: Biocompatibility; PHA; PHBHHx; PLA; Tissue engineering

1. Introduction

Polyhydroxyalkanoates (PHAs) are a family of polyesters synthesized by many bacteria as intracellular carbon and energy-storage compounds [1–4], and have attracted increasing interest as tissue engineering materials due to their adjustable physical properties, biodegradability and good biocompatibility [5–8]. More than 150 types of PHAs consisting of various monomers have been reported [9]. Among the PHA family, most studies have focused on poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxyburyrate-*co*-3-hydroxyvalerate) (PHBV) as biomaterials for in vitro and in vivo studies [10–12]. However, their brittleness, or low mechanical strength, has limited their application [13].

Recently, many studies have been published concerning poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx) as a potential implant material. Its mechanical properties and mode of manufacture have been shown to be improved over PHB and PHBV [14]. In vitro tests have demonstrated that PHBHHx has good biocompatibility for several cell types, including fibroblasts [15], chondrocytes [16] and osteoblasts [17]. Research has also shown that PHBHHx had better biocompatibility than poly(L-lactic acid) (PLA), PHB and PHBV [17,18]. However, most of the previous studies did not compare cell numbers between PHBHHx and tissue culture plates (TCPs) [17–25]. One study showed that cell growth on PHBHHx film was not as good as that on TCPs [26]. Another study demonstrated that the cell number on PHBHHx film was equal to that on TCPs [27]. These results revealed that PHBHHx could support the growth of various cells but was no better at increasing the cell number than TCPs. Obviously, it is necessary to continue the search for new biomaterials with better mechanical and thermal

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properties as well as good biocompatibility to meet the needs of different medical implants. A recent study has shown that poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) (PHBVHHx) has higher thermal stability, flexibility and mechanical strength than PHBV and PHBHHx [28], and could be a good biomaterial for tissue engineering.

Bone marrow MSCs are pluripotent stem cells, have the capacity for self-renewal with a high proliferative potential and can differentiate into multiple mesenchyme-lineage cell types, including osteoblasts, chondrocytes, adipocytes, myoblasts and stroma [29]. Studies involving a variety of animal models have shown that MSCs could be useful in the repair or regeneration of damaged bone, cartilage or myocardial tissues, and are an attractive stem cell source for the regeneration of damaged tissues in clinical applications [30–32]. Bone marrow MSCs are considered to be one of the most promising cell types in the field of tissue engineering [26]. Some studies have tried to regenerate bone tissue by combining polymer biomaterials with bone marrow MSCs [17,22,23].

In this study, PHBVHHx was produced by recombinant *Aeromonas hydrophila* 4AK4 harboring the PHA synthesis genes *phaAB* using propionic acid and dodecanoic acid as carbon sources. For the first time, this study examines the effect of PHBVHHx on growth and osteogenic differentiation of human bone marrow MSCs.

2. Materials and methods

2.1. Production of PHBVHHx

Wild-type *A. hydrophila* 4AK4 does not contain the *phaAB* genes encoding β-ketothiolase (*phaA*) and NADPH-dependent acetoacetyl-CoA reductase (*phaB*) that are essential for forming 3-hydroxyvaleryl-CoA (3HV-CoA). Construction of the recombinant *A. hydrophila* 4AK4 harboring *phaA* and *phaB* (*phaAB*) genes was previously described [33]. The recombinant strain was prepared using plasmid pTG01 harboring *pha*A and *pha*B cloned from plasmid pUC-AB. This strain was grown at 30 °C in mineral medium supplemented with 8 g l^{-1} dodecanoic acid and 6 g l^{-1} propionic acid as carbon sources. The mineral medium and trace elemental solution were the same as described [34]. The mineral salt medium consisted of (g l^{-1}) 9 $Na_2HPO_4{\cdot}12H_2O$, 1.5 KH_2PO_4, 1 $(NH_4)_2SO_4$, 0.41 $MgSO_4{\cdot}7H_2O$, 0.05 Fe(III)NH_4–citrate, and 0.02 $CaCl_2{\cdot}2H_2O$. In addition, 1 ml of trace element solution was added to the medium. The trace element solution contained: 100 mg $ZnSO_4{\cdot}7H_2O$, 30 mg $MnCl_2{\cdot}4H_2O$, 300 mg H_3BO_3, 200 mg $CoCl_2{\cdot}6H_2O$, 10 mg $CuSO_4{\cdot}5H_2O$, 20 mg $NiCl_2{\cdot}6H_2O$ and 30 mg $NaMoO_4{\cdot}2H_2O$ in 1 l of 0.5 N HCl. Kanamycin was added to the medium at a final concentration of 50 mg l^{-1} to maintain the stability of the plasmid. The cultures were incubated in 500 ml conical flasks containing 100 ml culture broth in a rotary shaker (FUMA QYC2112, Shanghai, China) at 200 rpm and 30 °C for 60 h. P(3HB-*co*-3.9 mol.% 3HV-*co*-13.4 mol.% 3HHx) was produced by this recombinant strain and used in this study.

2.2. Preparation of polyester films

PLA was provided by Nature Work, USA. PHB was provided by Jiangsu Lantian Group (Jiangsu, China). P(3HB-*co*-5 mol.% 3HV) was provided by Zhejiang TianAn Biomaterials, China. P(3HB-*co*-12 mol.% 3HHx) was provided by Lukang Co. Ltd. (Shandong, China). To prepare polyester films, 1 g of material was dissolved in 50 ml of chloroform, which was then poured into Petri dishes. The dishes were maintained at room temperature for 24 h to allow the complete evaporation of chloroform. Vacuum drying was applied to completely remove any possible solvent remaining in the films.

2.3. Scanning electron microscopy examination

Cells were treated for scanning electron microscopy (SEM) by following the published method [22]. The specimens were washed twice with phosphate-buffered saline (PBS) before being immersed in PBS containing 5% glutaraldehyde (pH 7.4) for 1 h. They were then dehydrated in increasing concentrations of ethanol (30, 50, 70, 90, 95 and 100%), followed by lyophilization. Next, they were mounted on aluminum stumps coated with gold in a sputtering device for 1.5 min at 10 mA and examined under a scanning electron microscope (JSM-6360, JEOL, Japan).

2.4. Atomic force microscopy analysis

Atomic force microscopy (AFM) images were obtained to analyze surface roughness as previously described [35] (Nanoscope IIIa Multimode, Digital Instruments, Santa Barbara, CA, USA). The resonance frequency was 344–400 kHz. The root mean square (rms) roughness (R_q), mean roughness (R_a) and maximum roughness (R_{max}) of the polymer surface were calculated based on a standard formula integrated in the software. The sampling areas were 3 μm × 3 μm. The values of five different spots per film were measured and averaged.

2.5. Thermal analysis

Differential scanning calorimetry (DSC) was used to analyze thermal transitions of polymers on Q-100 DSC (TA, USA) under a nitrogen atmosphere. Samples were heated from −60 to +180 °C at a heating rate of 10 °C min^{-1}. After maintaining this temperature for 1 min, the samples were rapidly quenched to −60 °C and process was repeated.

Thermogravimetry (TG) analysis was carried out with Q50 TGA (TA, USA). The mass of each specimen was 3–5 mg, and the reaction environment was flowing nitrogen. The rate of temperature rise was 10 °C min^{-1}. TG

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