

Characteristic cell adhesion behaviors on various derivatives of poly(3-hydroxybutyrate) (PHB) and a block copolymer of poly(3-[RS]-hydroxybutyrate) and poly(oxyethylene)



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ABSTRACT

A high-molecular-weight ABA tri-block copolymer ([RS]-PHB/PEG), consisting of poly(3-[RS]-hydroxybutyrate) ([RS]-PHB: A) and poly(oxyethylene) (polyethylene glycol; PEG: B), was synthesized by ring-opening polymerization of [RS]- β -butyrolactone ([RS]- β BL) in the presence of PEG and 1,3-dichlorotetrabutylidistannoxane (DTD) as the catalyst. By using a series of PHB derivatives including the new block copolymer, cell adhesion was evaluated with 3T3 fibroblast cells. It was found out that the attached cells didn't extend on [RS]-PHB, but extended on [R]-PHB although the numbers of the attached cells were very small on both [RS]-PHB and [R]-PHB. [RS]-PHB/PEG exhibited even poorer cell adhesion than [RS]-PHB because of the increased hydrophilic nature due to the PEG segments. Although the amount of fibronectin adsorbed on the surface of [RS]-PHB was higher than that on the surface of [R]-PHB, the cell adhesion became poorer on the fibronectin-pre-adsorbed [RS]-PHB. These differences in cell adhesion among these PHB derivatives were reasonably attributed to their surface properties related with the accumulation of methyl groups.

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1. Introduction

Various aliphatic polyesters are now applied as biomedical materials such as sutures, matrices for drug delivery, and scaffolds for tissue engineering. Among them, polyglycolide (PGA) and polylactide (PLA) have attained a practical utility as bioabsorbable materials due to their excellent biodegradability [1–7]. On the other hand, microbial polyesters, poly(3-[R]-hydroxyalkanoate)s (PHA), have little been used in biomedical fields because of their poor biodegradability in human body. However, their excellent biocompatible nature has long inspired prospect for their use as biomedical materials, although little study has been done until now [8]. Various PHB derivatives such as poly(3-[R]-hydroxybutyrate-co-3-[R]-hydroxyvalerate) ([R]-PHB/HV) have been bacterially synthesized until now, and the relation between the unit composition and cell adhesion of these modified [R]-PHB derivatives has been investigated [9,10]. The optically inactive (racemic) poly(3-[RS]-hydroxybutyrate) ([RS]-PHB), in return, can only be

synthesized by chemical process depending on the ring-opening polymerization (ROP) of racemic [RS]- β -butyrolactone ([RS]- β -BL) [11–15]. Being different from [R]-PHB, it is an elastic polymer and once attracted industrial attention for use as a biodegradable thermoplastic elastomer [16–24]. However, its real application has not yet been accomplished, probably because of the deficient data on the properties. We have recently shown that this [RS]-PHB exhibits a particularly low cell adhesion so that the cells placed on its surface are likely to keep a round shape without cell proliferation or extension occurring [25]. This behavior can be retained even when fibronectin, a cell-adhesion protein, is pre-adsorbed on the surface. We also synthesized poly(3-[RS]-hydroxybutyrate-co-glycolate-co- α -[S]-malate) ([RS]-PHB/GM) by the ring-opening copolymerization (ROCP) of [RS]- β BL and a functional monomer, 3-(S)-[(benzyloxycarbonyl)methyl]-1,4-dioxane-2,5-dione (BMD) [26] consisting of both glycolate (GA) and β -benzyl α -[S]-malate (BM) units, followed by the catalytic hydrogenolysis to remove the benzyl protecting groups [27]. The carboxyl pendants of the [RS]-PHB/GM was demonstrated to act as crosslinking points and other functionalization sites.

In this study, we synthesize an ABA tri-block copolymer ([RS]-PHB/PEG) consisting of [RS]-PHB (A) and poly(oxyethylene)

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(polyethylene glycol; PEG; B) by the controlled ROP of [RS]- β BL in the presence of PEG and 1,3-dichlorotetrahydroxydistannoxane (DTD) as the initiator and catalyst, respectively (Scheme 1). Since this block copolymer, having a high molecular weight, can be fabricated into self-supported polymer film, we study its cell adhesion and surface properties together with those of a series of PHB derivatives to analyze the specific character of [RS]-PHB and the effect of the hydrophilic PEG chains on the surface properties of [RS]-PHB. Based on these studies, the relationship between the surface chemistry and cell adhesion of the [R]- and [RS]-PHB derivatives are discussed.

2. Experimental

2.1. Materials

[RS]- β -BL was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and purified by repeated distillation over calcium hydride at reduced pressure before use. DTD was prepared from dibutyltin dichloride (Nacalai Tesque Co., Kyoto, Japan) according to the literature [27,28]. It was recrystallized from n-hexane before use ($T_m = 113\text{--}116\text{ }^\circ\text{C}$). A mono-dispersed poly(ethylene glycol) (PEG) having a number-average molecular weight (M_n) of 20,000 and a number- and weight-average molecular weight (M_w) ratio $M_w/M_n = 1.07$ and albumin (form bovine serum, crystalline) were purchased from Wako Chemical Co. (Kyoto, Japan). Tin octoate (SnOct_2) was purchased from Nacalai Tesque Co. (Kyoto, Japan) and purified by distillation under high vacuum. It was dissolved in distilled toluene in a concentration of 0.1 g/ml. [RS]-PHB was chemically synthesized according to the literature [25]. [R]-PHB having a 99.4 % ee in unit optical purity was supplied by Takasago Koryo Co. Ltd. (Tokyo, Japan). [R]-PHB/HV (Biopol[®]) was supplied by Nippon Monsanto Co. Ltd. (Tokyo, Japan). 3T3-L1 fibroblast cells were derived from NIH Swiss fetal mouse.

2.2. Measurements

200 MHz ^1H NMR spectra were measured on a Varian (USA) Gemini-200 spectrometer in CDCl_3 containing 1 vol-% tetramethylsilane (TMS) as the internal reference. The number- (M_n) and weight-average (M_w) molecular weights were determined by gel permeation chromatography (GPC). The analyzer was composed of a Shimadzu (Kyoto, Japan) LC-10A pump, a Shodex RI SE-31 RI detector, and a Shimadzu C-R7A Chromatopac data processor. A combination of two polystyrene gel columns of Tosoh (Tokyo, Japan) TSK gel G4000H₈ and G2500H₈ (7.5 mm I.D. \times 300 mm, each) was used with chloroform as the eluant at 35 $^\circ\text{C}$. The molecular weights

were calibrated according to polystyrene standards. Differential scanning calorimetry (DSC) of polymeric products was conducted on a Mac Science (Tokyo, Japan) DSC-3100 thermal analyzer under a nitrogen flow of 20 ml/min at heating and cooling rates of 10 $^\circ\text{C}/\text{min}$, using 2.0 mg of samples with α -alumina as the reference sample. The melting temperature (T_m) was taken as the peak temperature of the melting endotherm in the first scan. The glass transition temperature (T_g) was taken as the inflection point of the specific heat decrement at the glass transition of the samples quenched in liquid nitrogen in the second scan. Ultraviolet spectroscopy (UV) was performed on a Shimadzu-1600 PC spectrometer. Fluorescence was measured on a Shimadzu-RF-1500 analyzer. Contact angles of polymer films against water were measured with a Kyowa Kaimen Kagaku (Tokyo, Japan) CBVP-A1 surface tension meter at 25 $^\circ\text{C}$ according to the Wilhelmy method [29,30]. The advancing (θ_a) and receding (θ_r) contact angles were calculated according to Eqs. (1) and (2);

$$F_a = \gamma L \cos \theta_a \quad (1)$$

$$F_r = \gamma L \cos \theta_r \quad (2)$$

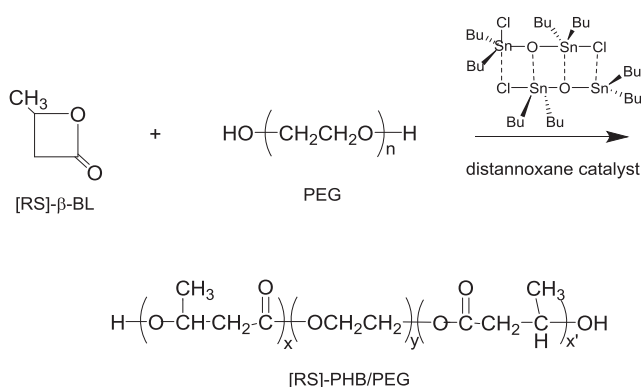
where F_a and F_r denote the forces at zero depth of immersion of the sample film at advancing and receding scans, respectively, while γ and L are the surface tension of the tested liquid and the peripheral length of the sample film, respectively. Atomic force microscopy (AFM) was conducted on a Digital Instrument (USA) Nanoscope-IIIa instrument operated in air using a silicon cantilever. A polymer film with a size of $1 \times 1\text{ cm}^2$ was equipped with an AFM stage to measure the surface roughness in tapping mode. FT-IR spectra were recorded on a JASCO FT/IR-5300 spectrometer fitted with JASCO (Tokyo, Japan) ATR-500/M adhesion in the wavenumber range of $4600\text{--}400\text{ cm}^{-1}$. ATR-FTIR analyses were performed with an internal reflection element composed of a 45°KRS-5 plate or a 45°Ge plate.

2.3. Synthesis of [RS]-PHB/PEG

In a round-bottomed flask, PEG (1.0 g, $5.0 \times 10^{-4}\text{ M}$) was mixed with benzene (2 ml), lyophilized, and dried *in vacuo* at 90 $^\circ\text{C}$ for 4 h. Then, DTD (222 mg, 10.2 mM) was added to it, and the mixture was dried again *in vacuo* at 90 $^\circ\text{C}$ for 4 h. To this mixture, [RS]- β BL (9.0 g, $1.04 \times 10^{-1}\text{ M}$) was added, and the reaction system was held at 100 $^\circ\text{C}$ for 4 h with stirring. The produced solid product was dissolved in a chloroform and precipitated into a suspension of diethyl ether and water (100: 1). The precipitates were filtered and dried *in vacuo*.

2.4. Synthesis of [RS]-PHB/GM

The carboxyl-functionalized [RS]-PHB/GM was synthesized according to the procedure we had previously reported (Scheme 2) [27]. In short, BMD (18.0 mg, 0.11 mM), [RS]- β BL (0.87 g, 10.1 mM), and DTD (11.5 mg, $10.4 \times 10^{-3}\text{ mM}$) were reacted in a sealed tube at 100 $^\circ\text{C}$ for 4 h. The resultant copolymer was dissolved in chloroform (3–4 ml) and precipitated into an excess amount of a diethyl ether/n-hexane (1:3 in volume) mixture, and the precipitates were separated, filtered, and thoroughly dried at 45 $^\circ\text{C}$ in vacuum to obtain poly(3-[RS]-hydroxybutyrate-co-glycolate-co- β -benzyl- α -[S]-malate) ([RS]-PHB/GBM). Then, a portion of [RS]-PHB/GBM (0.5 g) were suspended with palladium on charcoal (10%, 250 mg) in an ethanol/1,4-dioxane mixture (10: 90 in vol-%, 50 mL) and reacted with hydrogen gas under a vigorous stirring for 48 h. The solution, removed from the catalyst, was evaporated, and the residue was reprecipitated from chloroform (solvent) to an excess



Scheme 1. Synthesis of the triblock copolymer [RS]-PHB/PEG.

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