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Characterizations and Release Behavior of Poly [(R)-3-hydroxy butyrate]-*co*-Methoxy Poly(ethylene glycol) with Various Block Ratios

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Abstract: Poly[(R)-3-hydroxy butyrate] (PHB) and methoxy poly(ethylene glycol) (mPEG) were conjugated by the transesterification reaction with tin(II)-ethylhexanoate (Sn(Oct)-II) as a catalyst. Hydrophobic PHB and hydrophilic mPEG formed an amphiphilic block copolymer which was formed with the self-assembled polymeric micelle in aqueous solution. In this study, we tried to determine the optimum ratio of hydrophobic/hydrophilic segments for controlled drug delivery. The particle size and shape of the polymeric micelle were measured by atomic force microscopy (AFM) and transmission electron microscopy (TEM). Their size were 61-102 nm with various block ratios. Griseofulvin was loaded in the polymeric micelle as a hydrophobic model drug. The loading efficiency and release profile were measured by high performance liquid chromatography (HPLC). The model drug in our system was constantly released for 48 h.

Keywords: PHB, polymeric micelle, hydrophobic and hydrophilic block ratio, drug delivery.

Introduction

Poly[(R)-3-hydroxy butyrate], PHB was an aliphatic polyester produced by microorganism. It was also the first identified member of PHA family. In 1926, it was discovered by Lemoige in *Bacillus megaterium*.[1] It is very useful polymer because of the biodegradable and biocompatible properties.[2,3] It was possible to produce PHB by recombinant *Escherichia coli* in the large scale.[4] Many researchers studied PHB because of its biocompatibility, biodegradability and special chemical properties. In the biomedical field, PHB can be used as drug carriers, surgical sutures and the scaffolds for tissue engineering.[5,6] It is necessary to make the low molecular weight PHB by the hydrolysis method[7] because the high molecular weight one has brittle properties due to the high crystallinity.[8] PEG is the biocompatible material which was approved by the food and drug administration (FDA). PEG could be used in various fields. It played an important role especially in the biomedical field. It is hydrophilic, biocompatible and flexible polyether.[9-12] Poly(ethylene glycol), PEG could be modified to have functional groups which were ready to react with other functional groups.

In our previous study, PHB-*co*-mPEG diblock copolymer had been successfully conjugated by the transesterification reaction in the melt state.[7] PHB-*co*-mPEG diblock copolymer had the amphiphilic property. The amphiphilic block copolymer usually formed the polymeric micelles in aqueous solution by the self-assembly. Polymeric micelles have many advantages. It was very small, highly stable, highly water soluble and low toxic.[14,15] But it should avert the removal by reticuloendothelial system (RES).[16] It could be used as a passive targeting carrier using the enhanced permeability and retention (EPR) effect.

There were many loading procedures of the drug such as simple equilibrium, dialysis, O/W emulsion, solution casting and freeze-drying.[13] In this study, the dialysis method was used. Griseofulvin (GF) was incorporated into the polymeric micelle as a hydrophobic model drug. GF is the antifungal agent which was first isolated from a *Penicillium spp.* in 1939. It is insoluble in water, and used for the oral treatment of skin particularly against dermatophytes. It was effective when it was reached the skin and hair after the oral ingestion.[17,18] Drugs can be located into the core of particles and then be taken by the hydrophobic interaction.[19] The drug loaded particles are relatively stable for a long time in the blood stream since the hydrophilic mPEG was covered the outer shell.

In this study, PHB and mPEG were conjugated to form the amphiphilic diblock copolymer with various block length of PHB (3,500 and 5,000) and mPEG (750, 2,000 and 5,000). It was expected to have different particle sizes, critical micelle concentration (CMC) and release profiles. The formation of polymeric micelle in aqueous solution was

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verified by fluorescence spectrometer and transmission electron microscope (TEM).

Experimental

Materials. Poly[(R)-3-hydroxy butyrate] (PHB) was prepared by the hydrolysis method.[7] Monomethoxy poly(ethylene glycol) (PEG) (M.W.=750, 2,000 and 5,000) was purchased from Sigma-Aldrich. 1-Pyrene-carboxaldehyde (99%), tin(II)-ethylhexanoate, griseofulvin (GF)(>95% HPLC grade) were purchased from Sigma-Aldrich. Acetonitrile was HPLC grade and purchased from SK chemical. Deionized water (18 M) was made by the Millipore water system. Dialysis membranes were purchased from Spectra/Por®.

Synthesis and Characterization of Amphiphilic Diblock Copolymer. PHB (540 mg) and mPEG (550 mg) were added in 50 mL flask and temperature was raised to 180 °C in the vacuum condition. When they were melted after 15 min, Sn(Oct)-II(45.56 μL) was added in the flask under the argon environment. The reaction time was 30 min.[7] The reaction scheme of PHB-*co*-mPEG was showed in Figure 1.

The critical micelle concentration (CMC) was measured by the fluorescence spectrometer. The pyrene was dissolved in acetone and diluted in water (1.2×10^{-5} g/L). Then, acetone was evaporated. The polymer solutions were prepared with various concentrations from 0.1×10^{-4} to 0.5 g/L. Pyrene stock solution (100 μL) was added to 1,900 μL of each samples. Excitation spectra were recorded from 300 to 360 nm and emission wavelength was 390 nm.

The size and shape of particles were observed by atomic force microscope (AFM) (NanoscopeIIIa, Digital Instruments, USA) and transmission electron microscope (TEM, EM 912 Omega, KBSI). The samples were prepared by the freeze dryer.

Biodegradability Test. PHB (5,000), mPEG (5,000) and PHB-*co*-mPEG were prepared for the biodegradability test. 100 mg of each samples were immersed in phosphate buffer saline (PBS) solution (pH 7.4) including 0.1 g/L lipase 100T at 37.5 °C. PBS solution was entirely changed every 5 days. Samples were periodically collected and washed 3 times with the distilled water. The degree of biodegradation was measured by the change of molecular weight using gel permeation chromatography (GPC).

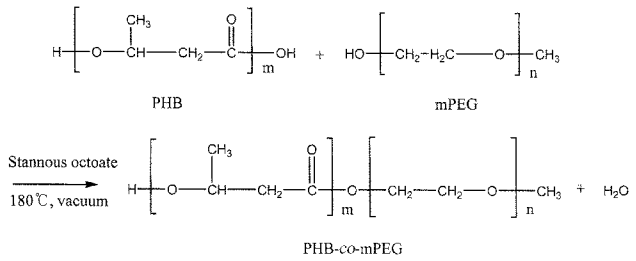

Figure 1. Reaction scheme of PHB-*co*-mPEG.

Drug Loading and Release Test. GF as a hydrophobic drug was used in this experiment. The dialysis method was used for the drug loading procedure. 5 mg of GF was dissolved in 10 mL water/acetonitrile (1/9) and homogenized for 20 min. 50 mg of PHB-*co*-mPEG diblock copolymer was dissolved in 10 mL of acetonitrile. Two solutions were mixed in the dialysis membrane for 24 h.

The drug loading efficiency was measured by high performance liquid chromatography (HPLC). C_{18} column (MU cleosil 100-5) with 5 μm particle size, 250 mm length and 4 mm inner diameter were used to detect the drug. The mobile phase was a mixture of 45 mM potassium acid solution and acetonitrile (45 vol%). Phosphoric acid solution was used as a buffer solution to maintain the pH at 3. The flow rate of mobile phase was 1 mL/min. The concentrations of GF in water/acetonitrile (1/9) were 0.00025~0.1 g/L to obtain the standard curve. The typical retention time of GF was 5 min which could be monitored at 293 nm.[20] The calculation is as follows[21]:

$$\text{Drug loading efficiency} = \frac{\text{actual amount of drug loaded in micelles}}{\text{theoretical amount of drug loaded in micelles}} \times 100(\%)$$

The release behavior of GF in the dialysis membrane (molecular weight cut off: 1,000, 5 mL) was examined in 95 mL PBS solution (pH 7.4) at 37 °C with the stroke of 70 rpm. The released media was collected at the regulated time intervals. After the sampling, entire PBS solution was replaced with the fresh PBS solution to maintain the sink condition. It was also estimated by HPLC.

Results and Discussion

Effects of the Hydrophobic/Hydrophilic Block Ratio. We reported the synthesis and characterization of PHB-*co*-mPEG diblock copolymers. The polymers with hydrophobic/hydrophilic segments could be formed the polymeric micelles by the self-assembly in aqueous solution. The hydrophilic segments formed the outer shell of micelle and the hydrophobic segments located in the inner core. The CMC and mean particle size of the PHB-*co*-mPEG diblock

Table I. The Block Ratio, Mean Particle Size and CMC Values of PHB-*co*-mPEG Diblock Copolymer

Samples	Molecular Block Ratio	M_n^a	Mean Particle Size[b](nm)	CMC (g/L)[c]
1	5,000-750	5,625	61	1×10^{-3}
2	5,000-2,000	6,850	78	5×10^{-3}
3	5,000-5,000	9,908	102	5×10^{-2}
4	3,500-5,000	8,383	86	1×10^{-2}

[a]Measured by GPC. [b]Measured by AFM. [c]Measured by fluorescence spectrometer.

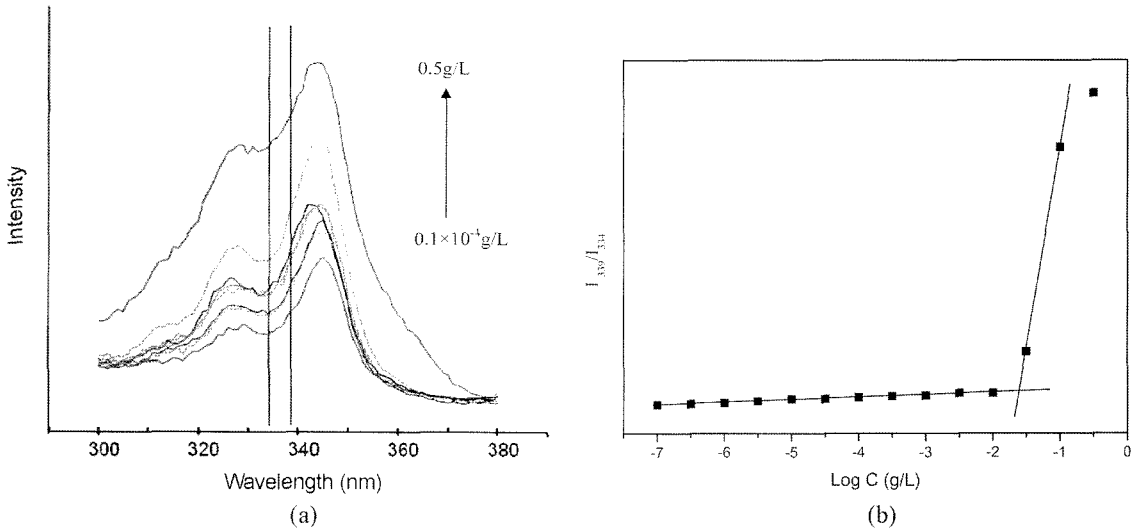

Figure 2. CMC measurement of PHB(5,000)-*co*-mPEG(5,000) at 24 °C. (a) Excitation spectra of pyrene with various concentration (0.1×10^{-4} ~0.5 g/L) and (b) Plot of fluorescence intensity (I_{339}/I_{334}) of pyrene vs logarithmic concentration.

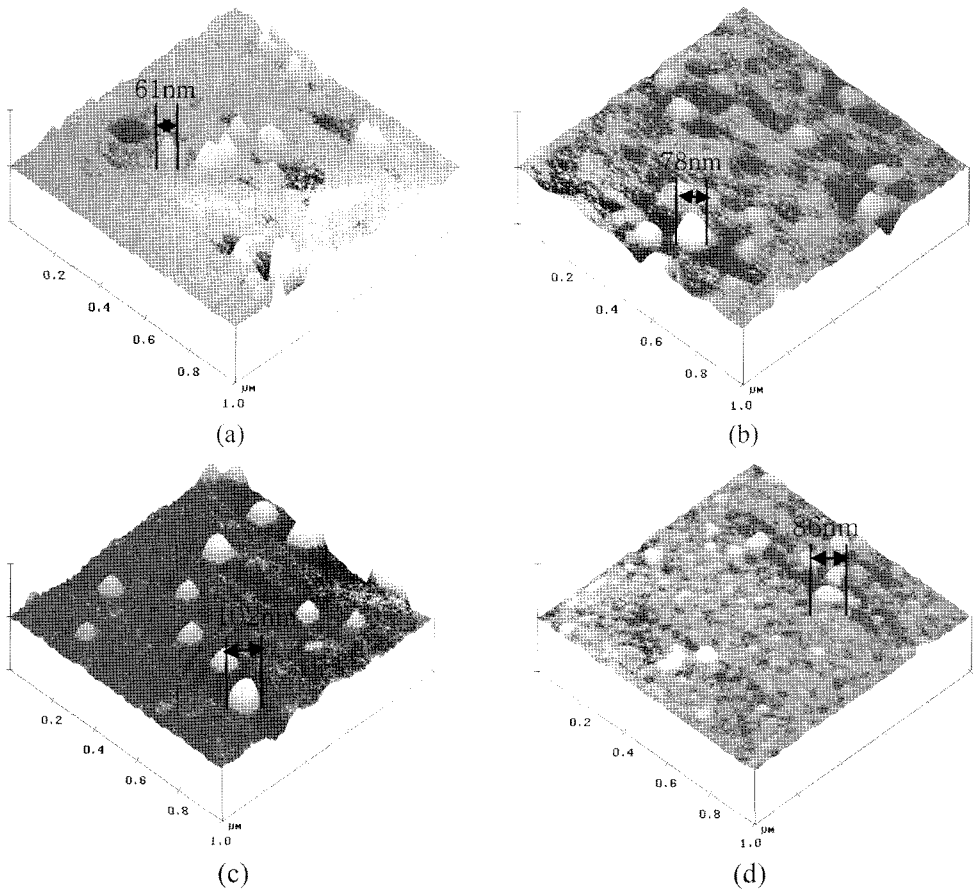

Figure 3. AFM images of PHB-*co*-mPEG. (a) sample 1, (b) sample 2, (c) sample 3, (d) sample 4.

copolymer are listed in Table I. PHB-*co*-mPEG diblock copolymer has formed the self-assembled particles even at the very low concentration. The CMC values of sample 1 was 1×10^{-3} g/L, sample 2 was 5×10^{-2} g/L and sample 3 was 5×10^{-2} g/L (Figure 2). Figure 2(a) is the pyrene excitation spectra of PHB-*co*-mPEG (0.1×10^{-4}~0.5 g/L). It might start to form the micelle structure above the CMC value. The longer the mPEG block length, the higher the CMC values

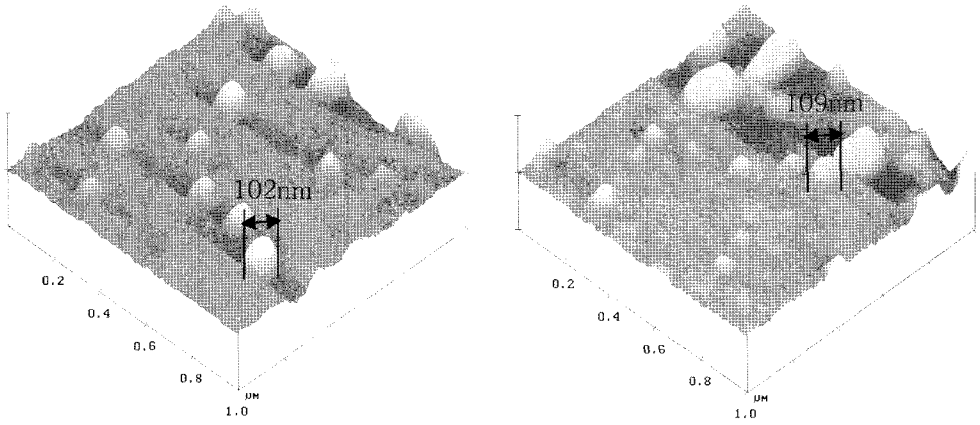

Figure 4. AFM images of sample 3. (a) blank micelle and (b) drug loaded micelle.

of PHB-*co*-mPEG was obtained. When the hydrophobic block length was relatively short, the CMC value was more increased. The CMC of sample 3 was smaller than sample 4. The stability of polymeric micelle was increased the decrease of CMC. The hydrophobic/hydrophilic block ratio and the incorporated drug amount could influence the CMC, size and shape of polymeric micelles. The stability of micelle was also increased with the increase of T_g.[22]

AFM and TEM images showed the shape as well as the size of the particles. The particle size of PHB-*co*-mPEG with various block ratios was varied from 61 to 102 nm (Figure 3). Sample 3 showed the biggest size (102 nm) among the various block ratios. The increase of hydrophilic segments might influence the mechanism of self-assembly which was mainly affected by the hydrophobic interaction. TEM image of sample 3 was also showed the spherical particle with about 100 nm diameters in Figure 5. When drug

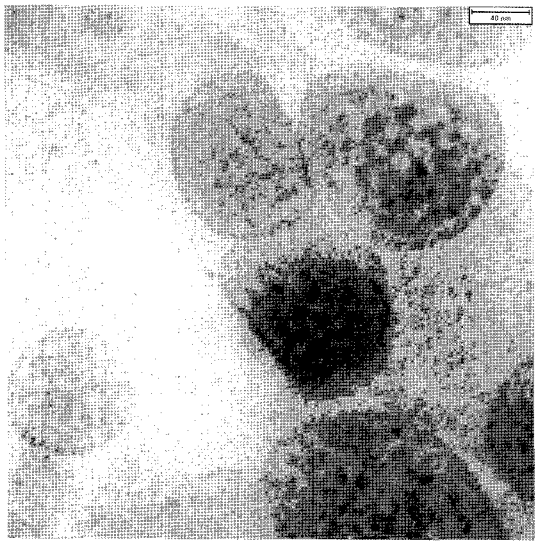

Figure 5. TEM image of sample 3.

Table II. Mean Particle Size and Drug Loading Efficiency of Drug Loaded Micelles

Samples	Molecular Block Ratio	Mean Particle Size[a] (nm)	DLE[b] (%)
1	5,000-750	68	39
2	5,000-2,000	82	67
3	5,000-5,000	109	25
4	3,500-5,000	93	14

[a]Measured by AFM. [b]Measured by HPLC.

was loaded in the polymeric micelles, mean particle size was bigger because the drug was occupied the inner part of polymeric micelles. Drug loaded micelle was bigger than the blank one (Figure 4). Mean particle sizes of drug loaded micelles were changed from 68 to 109 nm by block ratios (Table II).

Biodegradable Behavior of PHB-*co*-mPEG Diblock Copolymer. Biodegradable behavior was investigated in the PBS solution with lipase for 30 days. Figure 6 showed the changes of the molecular weight. All samples did not show any changes for 5 days. PHB and mPEG without lipase did not show any changes for 30 days. The PHB with lipase showed the slight decrease of molecular weight. mPEG has no biodegradability. The molecular weight of PHB-*co*-mPEG copolymer was decreased about 30% in the lipase added PBS solution after 30 days. The biodegradability of copolymers in the body could be controlled by the length of PHB segment.

***In vitro* Drug Release.** The sizes of polymeric micelles with drug were measured by AFM (Table II). The polymeric micelle with hydrophobic drug was bigger than the blank polymeric micelle. Table II showed the mean particle size and DLE of drug loaded micelles. DLE of sample 2 was the highest (67%) and that of sample 4 was the lowest

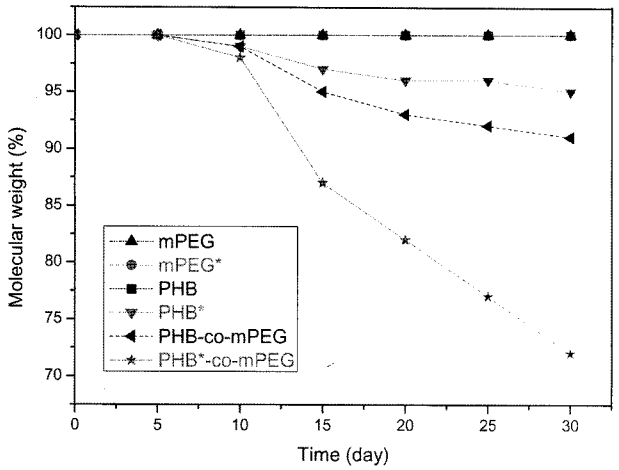

Figure 6. The change of PHB(5,000), mPEG(5,000) and PHB-*co*-mPEG copolymer in PBS solution at 37 °C (*lipase added).

(14%). Sample 3 could encapsulate the drug more than sample 4 because of the longer hydrophobic block. The longer hydrophobic segments are, the stronger hydrophobic interaction exists between polymeric micelle and drug. On the other hand, the hydrophilic segment like mPEG had interfered the flow of the drug into the polymeric micelle. Hence, polymeric micelle with longer mPEG block might encapsulate the lower amount of the hydrophobic drugs.

Figure 7 showed the *in vitro* release profiles of PHB-*co*-mPEG diblock copolymers. The release test was performed in the mimicking condition of biological system.[23] Drug release behavior of the sample 1 and sample 2 showed the initial burst effect. In sample 3 and sample 4, the drug release was sustained for 48 h. The hydrophobic segment of sample 3 was longer than sample 4. The hydrophobic interaction with drugs was strong with long hydrophobic segments.[24,25] Hence, the hydrophobic drug was diffuse out more slowly in sample 3 than sample 4. When the length of the hydrophobic block was maintained at the same, the hydrophobic drug was more slowly flow out with the longer hydrophilic segments. When the hydrophilic segment is longer, the hydrophobic drug is difficult to release because hydrophilic outer shell was surrounding the hydrophobic drug. The hydrophilic segment was strongly wrapped the hydrophobic group for the protection.

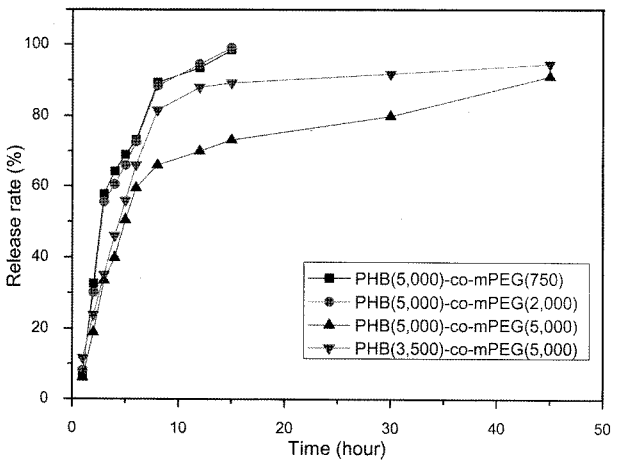

Figure 7. *In vitro* release profiles of GF from PHB-*co*-mPEG copolymers.

Conclusions

In this study, we controlled the block ratios of PHB and mPEG. They showed the different loading efficiencies and release profiles with different block ratios. Even if the longer hydrophilic segment might increase the size of particles, the release behavior showed the sustained release profile. The CMC values of our system had 5×10^{-3}~1×10^{-2} g/L. The sizes of the spherical particles was 61~102 nm. The size of drug loaded micelle was 68~109 nm. Drug loading efficiencies of our system ranged between 14~67% with various hydrophobic-hydrophilic block ratios. It was possible to design the drug carrier which has the high drug loading efficiency and sustained release profile. Our polymeric micelles could be used as a passive targeting drug delivery system.

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