[SPECIAL EDITION on Annual Meeting and Symposium 2017–Transaction]

Effect of Molecular Weight on Physical and Crystallization Properties of UHMW-poly [(*R*)-3-hydroxybutyrate-co-(*R*)-3-hydroxyhexanoate]

Taizo Kabe^{*1,2,3,4}, Takashi Sugiura^{*1}, Takaaki Hikima^{*5},
Masaki Takata^{*4}, and Tadahisa Iwata^{*1,3,4,#}

^{*1}Science of Polymeric Materials, Graduate School of Agricultural and Life Sciences, The University of Tokyo,
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

^{*2}Materials Structure Group I, The Research and Utilization Division, Japan Synchrotron Radiation Research Institute (JASRI),
1-1-1 Kouto, Sayo-cho, Sayo-gun, Hyogo 679-5148, Japan

^{*3}Japan Science and Technology Agency, CREST, 4-1-8, Honcho Kawaguchi, Saitama 332-0012, Japan

^{*4}Materials Visualization Photon Science Group, RIKEN SPring-8 Center,
1-1-1 Kouto, Sayo-cho, Sayo-gun, Hyogo 679-5148, Japan

^{*5}Research Infrastructure Group, RIKEN Harima Institute/SPring-8 Center,
1-1-1 Kouto, Sayo-cho, Sayo-gun, Hyogo 679-5148, Japan

Abstract: Ultra-high-molecular-weight poly[(*R*)-3-hydroxybutyrate-co-3.2 mol%-(*R*)-3-hydroxyhexanoate] (UHMW-PHBH), with a weight-average molecular weight (M_w) of approximately 400×10^4 , is extracted from genetically engineered microbial cells via an optimized solvent extraction method. PHBH polymers with various molecular weights (various MW-PHBHs) are prepared from the UHMW-PHBH using H_2SO_4 treatment. The thermal properties and spherulite growth morphology of the UHMW-PHBH and various MW-PHBHs are analyzed in order to investigate the properties of UHMW-PHBH and the effect of the molecular weight on the above properties. The value of T_m and T_g were same regardless of M_w above 62×10^4 . The crystallization half time ($t_{1/2}$), as an index of overall crystallization speed, of UHMW-PHBH did not change, compared with that of normal molecular weight PHBH. The observation of crystallization morphology revealed that molecular chains with high-molecular-weight lead to decrease the growth rate of spherulite and increase the crystal nuclei forming frequency. Drawn films of UHMW-PHBH are prepared by one-step or two-step cold-draw methods, and their mechanical properties and highly ordered structure are analyzed by tensile tests and X-ray measurements, respectively. One-step cold-drawn UHMW-PHBH films exhibit enhanced properties relative to those of normal-molecular-weight PHBH. Wide-angle X-ray diffraction and small-angle X-ray scattering reveal that the one-step cold-drawn UHMW-PHBH possesses the rare β -form crystal structure. Additionally, the β -form crystal are increased drastically after two-step drawing process with increasing tensile stress. The crystal structure of those films were also investigated by synchrotron X-ray.

(Received 31 July, 2017; Accepted 14 November, 2017)

1. Introduction

Polyhydroxyalkanoate (PHA) is a type of polyester produced from renewable biological resources by a number of microorganisms[1–3]. PHA has a number of chemical structures depending on the carbon resource, strain and other factors[4–7]. Poly[(*R*)-3-hydroxybutyrate] (P(3HB)) is a prominent microbial polyester typical of the PHA family. P(3HB)

is brittle and weak due to secondary crystallization at room temperature, which is caused by the glass-transition temperature (T_g) being lower than the room temperature[8, 9]. Tensile strength, elongation at break and Young's modulus for this polymer are 10 MPa, 5%, and 0.5 GPa, respectively. Therefore, practical use of P(3HB) has been thought difficult, and many researchers have study ways to improve brittleness and improve the hardness properties. It

corresponding author: Tadahisa Iwata (E-mail: atiwata@mail.ecc.u-tokyo.ac.jp)

was found that the brittleness due to high crystallinity could be improved by introducing a second monomer. Poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] [P(3HB-*co*-3HH)] that is microbial synthesized from palm oil is a P(3HB) copolymer that includes a 3-hydroxyhexanoate (3HH) unit. The 3HH unit reduces the high crystallinity of P(3HB), and it prevents secondary crystallization. Furthermore, P(3HB-*co*-3HH) shows enhanced flexibility, with an elongation at break above 500 %, compared with that of P(3HB) which is <10 %. However, an improvement of the tensile strength of these P(3HB-*co*-3HH) has not been reported. For P(3HB), this issue has been resolved by ultra-high-molecular-weight P(3HB) [UHMW-P(3HB)] [with a weight-average molecular weight (M_w) above 300×10^4 , in contrast to the M_w of wild-type P(3HB) of around 60×10^4], which is biosynthesized by recombinant micrograms. The tensile strength of UHMW-P(3HB) film is about 70 MPa compared with about 10 MPa for wild-type P(3HB) one.

Drawing methods for P(3HB) and its copolymers films have also been investigated by many researchers. Optimum drawing methods lead to an enhancement of the mechanical properties. For example, cold-drawn P(3HB) film with a draw ratio of 10[10], two-step cold-drawn P(3HB-*co*-5 mol%-3HH) film with a draw ratio of 8[11], and the two-step hot-drawn UHMW-P(3HB) film with a draw ratio of 12[12], exhibit tensile strengths of 195 MPa, 140 MPa and 388 MPa, respectively.

Recently, the ultra-high-molecular-weight poly [(*R*) - 3 - hydroxybutyrate - *co* - 3.2 mol % - (*R*) - 3 - hydroxyhexanoate] (UHMW-PHBH) has been biosynthesized by genetically engineered microorganisms. However, details on UHMW-PHBH, such as a thermal properties, mechanical properties and crystal growth morphology have yet to be reported. Moreover, information on the basic properties of ultra-high-molecular-weight polyesters is poor because their rarity. The aim of this study is to measure the basic properties of UHMW-PHBH and investigate the dependence of those properties on the molecular weight. Additionally, a drawing method is applied to UHMW-PHBH in order to improve its mechanical properties.

2. Experimental

2.1 Extraction of UHMW-PHBH from microbial cells

Dried recombinant *Aeromonas caviae* cells that contain UHMW-PHBH biosynthesized from palm oil were provided by Kaneka Co. (Japan). The second monomer ratio of obtained UHMW-PHBH and various-molecular-weight PHBH are estimated at 3.2 mol% by ¹H-NMR. These dried cells (1 g) were mixed with chloroform (2 L), and the mixture was stirred at room temperature for 2 h by a mechanical stirring machine with stirring speeds of 10 or 400 rpm. The mixture was then filtered using filter grades No. 5C, No. 101, and No. 5A (Advantec, Toyo Roshi Kaisha, Ltd. Japan), with nominal pore size ratings of 1 μm, 5 μm, and 7 μm, respectively. The filtrate was put in hexane, and the UHMW-PHBH precipitate was separated. The extraction scheme are shown in Figure 1.

Fig. 1 Experimental scheme of extracting UHMW-PHBH from microbial cell.

2.2 Purification and molecular weight control

The extracted UHMW-PHBH was purified by re-dissolving in chloroform and re-precipitating using hexane or methanol. This procedure was performed three times to obtain polymers of the requisite purity. The purified UHMW-PHBH was then subjected to sulfuric acid (H_2SO_4) treatment in order to produce a series of PHBH polymers with a reduced molecular weight. For this procedure, a UHMW-PHBH/chloroform 1% solution (20 ml) was added to a sulfuric acid/tetrahydrofuran (0.05 ml / 0.45 ml) solution (5 ml), which was then stirred for different durations. The mixture was subsequently neutralized by adding an

aqueous saturated sodium bicarbonate solution. At this stage, two separate layers formed: an aqueous layer with the sodium salts, and an organic layer that contains the sulfuric acid-degraded UHMW-PHBH. The aqueous layer was removed from the solution mixture, and the organic layer was mixed with hexane and washed thoroughly. The molecular weights of the various-molecular-weight PHBH polymers (various-MW PHBHs) formed by sulfuric acid treatment were determined by using gel permeation chromatography (10 A series, Shimadzu Co., Japan) at 40 °C. The instrument was equipped with two joined columns of Shodex K-802 and K-806 M. Polystyrene standards were used for molecular weight calibration. The molecular-weight-controlled PHBH polymers obtained by this method were named according to their molecular weight.

2.3 Preparation of one- and two-step-drawn films of UHMW-PHBH

UHMW-PHBH films were prepared by the solvent-casting method from a chloroform solution. Next, one UHMW-PHBH film was melted at 170 °C for 20 s. Subsequently, the melted UHMW-PHBH film was quenched in an ice-water-bath, and it became an amorphous film. The amorphous UHMW-PHBH film was then drawn by a handmade drawing machine while still in the ice-bath. The drawn film was then annealed at 80 °C for 2 h. The film produced using this method is named the 'one-step cold-drawn film.' The two-step cold-drawn PHBH film involves the same process until the drawing in the ice-water-bath. At this stage, the one-step cold-drawing ratio was fixed at 8. The one-step cold-drawn film was annealed in an oven at 80 °C for 1 h, and then this annealed film was further drawn again. The second drawing process was conducted at room temperature. The drawn film obtained from the second drawing process was again annealed at 80 °C for 2 h, and is named the 'two-step cold-drawn film.'

2.4 Analysis methods

The thermal properties of the UHMW-PHBH and various-MW PHBHs were measured by differential scanning calorimetry (DSC) (DSC 8500, PerkinElmer, USA) under a nitrogen atmosphere. In the first run, the samples were heated at 20 °C/min from -70 °C to 210 °C, and subsequently quenched at -200 °C/min back to -70 °C. In the second run, the samples (after quenching) were heated again at 20 °C/min from -70 °C to 210 °C. The T_g , cold crystallization temperature (T_c), and melting temperature (T_m) were

calculated from DSC results obtained in the second run.

Crystal growth morphology of the UHMW-PHBH and various-MW PHBHs was observed via a polarization microscope (Nikon, ECLIPSE E 600 POL) equipped with crossed polarizers. The samples were melted at 200 °C for 1 min on a temperature-jump stage (Japan High Tech., LK 300 A). Subsequently, they were quenched at various crystallization temperatures (70, 80, 90, 100 or 110 °C), and held at that temperature until the crystallization was complete. The spherulite growth rates of the samples were calculated from the polarization microscopy images obtained during their growth. The crystallization half times were measured by DSC using the isothermal crystallization process, which are identical to heating profiles of polarization microscope observation. In this process, exothermic peak caused by crystallization are measured by DSC, and it convert to time integration value. The crystallization half time is decided as the half time against finish time till constant time integration value.

Thermal degradation kinetics of the polymers were calculated from the molecular weights of the melted samples. Samples of the UHMW-PHBH were melted on a hot-stage at pre-determined treated temperatures (170, 180, 190 or 200 °C) for given time periods (20, 40, 60, 120, 180, 240 or 300 s). Subsequently, the molecular weights of these samples, considered to be thermally degraded, were measured by gel permeation chromatography (GPC). The degree of polymerization (P_n) was calculated from the average molecular weight (M_n), which was plotted as a function of melting time to determine the thermal degradation constant (K_d) from the slope of the plot. These characterization methods are described in detail in References[13,14].

Mechanical properties of the drawn films were measured using a tensile testing machine (Shimadzu, EZ-test). The gauge length and cross-head speed were selected to be 10 mm and 20 mm/min, respectively.

Wide-angle X-ray diffraction (WAXD) and small angle X-ray scattering (SAXS) were carried out in the synchrotron radiation facility of SPring-8 Harima, Japan at BL-45 XU or BL-03 XU. Pilatus 2M or 1M were used as a detector. The camera length was calibrated by using Si(111) powder, and the wavelength was chosen to be 0.1 nm. The obtained data were analyzed using Fit2d software.

3. Results and discussion

3.1 Extraction of UHMW-PHBH from dry cells

A number of methods for extraction of PHA from dry cells are reported in literature, and each method has many process parameters. The organic solvent extraction method utilized in this study includes stirring the dry cells in chloroform followed by filtering in order to remove the cellular residue. It is possible that the fast stirring speeds and small pore sizes of the filters leads to lower-molecular-weight polymers being preferentially purified. To investigate this problem, extraction experiments under six different combinations of stirring speed and filter pore size were carried out. The results, given in Table 1 and Figure 2, show that the average molecular weight of the extracted UHMW-PHBH progressively decreases under the following stirring speed-filter pore size combinations: 400 rpm-7 μm , 10 rpm-7 μm , 400 rpm-5 μm , 10 rpm-5 μm , 400 rpm-1 μm , and 10 rpm-1 μm . The M_w under 400 rpm-7 μm was determined to be 420×10^4 , and the difference of molecular weights between 400 rpm-7 μm and 10 rpm-1 μm is approximately 50×10^4 (Table 1 and Figure 2). From these results, it can be concluded that the influence of stirring speed on the molecular weight is relatively small, and the filter pore size influences to pass through the highest M_w of PHBH. In normally, it is considered that the high stirring speed terminates molecular chain due to entanglement and

Table 1 Weight-average molecular weights with different extraction conditions.

Sample	$M_w \times 10^{-4}$	$M_n \times 10^{-4}$	M_w/M_n
High speed • Cotton plug	396	241	1.64
Low speed • Cotton plug	397	249	1.34
High speed • 7 μm	417	269	1.55
Low speed • 7 μm	409	243	1.69
High speed • 5 μm	407	257	1.59
Low speed • 5 μm	406	248	1.64
High speed • 1 μm	380	212	1.38
Low speed • 1 μm	371	201	1.40

Fig. 2 Weight-average molecular weights with different extraction conditions.

pulling between molecular chains in solution. However, this conclusion suggests that the filter pore size is important in the extracting UHMW-PHBH, which is probably due to the large radius of gyration of UHMW-PHBH in the chloroform solution.

3.2 Molecular weight control

A wide range of various-MW PHBH samples were prepared by sulfuric acid treatment. Molecular weights of the various-MW PHBH polymers were determined by GPC, and the results are summarized in Table 2. The inverse molecular weight of the various-MW PHBHs is a linear function with respect to the time of H_2SO_4 treatment, which indicates that the reduction of molecular weight is a result of random cutting by H_2SO_4 treatment.

3.3 Change in thermal properties as a function of molecular weight

Thermal properties such as T_g , T_c and T_m of UHMW-PHBH and various-MW PHBHs were determined by DSC as described earlier in the experimental section, and are summarized in Figure 3

Fig. 3 Thermal properties of various-MW PHBHs as a function of molecular weight.

Table 2 Relationship between sulfate treatment time and molecular weight of UHMW-PHBH. And thermal properties of various-MW PHBHs.

Reaction time [min]				First run		Second run			
	$M_w \times 10^{-4}$	$M_n \times 10^{-4}$	M_w/M_n	T_m [$^{\circ}\text{C}$]	ΔH_m [J/g]	T_g [$^{\circ}\text{C}$]	T_c [$^{\circ}\text{C}$]	ΔH_c [J/g]	ΔH_m [J/g]
0	400	250	1.6	156	92	4	58	61	158
8	140	63	2.2	155	64	2	61	41	156
15	99	50	2	154	64	4	63	44	157
30	62	31	2	155	71	3	61	48	157
45	46	24	1.9	155	63	3	59	42	156
60	32	18	1.8	156	71	-6	58	52	157
120	14	8	1.8	157	67	-1	58	49	147
240	8	5	1.6	152	14	-13	49	44	129
480	4	2	2	153	11				
960	3	2	1.5						

^a This value has not been measured by DSC.

^b This value was not detected in second run process by DSC.

and Table 2. The T_g of the $M_w=62\times10^4$ polymer, which corresponds to the wild-type PHBH (PHBH), was determined to be 4 °C, in agreement with values reported in literature. The T_g values were observed to decrease with decreasing molecular weight to temperatures lower than 4 °C. A similar phenomenon has been reported in polystyrene[15], and is attributed to the increase in the free volume within the polymer caused by the increasing number of chain end groups in low-molecular-weight PHBH. In contrast, the T_g values of polymers with $M_w > 62\times10^4$ show a constant value of approximately 4 °C. The T_c values increases with increasing M_w from $M_w=2.7\times10^4$ to $M_w=62\times10^4$, and decreases thereafter. The high T_c values at high M_w values are expected to be related to a crystallization mechanism dependent on the molecular weight. The T_m values show a drastically increase up to $M_w=32\times10^4$, and then gradually increase with increasing molecular weight, which is attributed to the increasing lamellar thickness.

3.4 Crystal growth morphology of UHMW-PHBH and various-MW PHBHs

Figure 4A show the crystallization half-times ($t_{1/2}$) of UHMW-PHBH ($M_w=400\times10^4$) and various-MW PHBHs ($M_w=140\times10^4$, 99×10^4 , and 46×10^4) as a function of isothermal crystallization temperature (The detail value is listed in Table 3). Here, a sample is considered to have a fast crystallization speed if it exhibits a short $t_{1/2}$ value. All samples have a minimum $t_{1/2}$ value larger than 1.4 min at the isothermal crystallization temperature around 70 °C. The minimum $t_{1/2}$ of the P(3HB) homopolymer is reported to be 1 min,[16] and the longer $t_{1/2}$ s of PHBHs than that of P(3HB) competition of the 1.4 min of PHBHs and 1 min of P(3HB) due to inhibition of crystallization by second monomer unit[17]. Comparison of the $t_{1/2}$ values with respect to the molecular weight shows that the former is not dependent on the this result.

Figure 4B shows the typical POM graphics of the UHMW-PHBH and various-MW PHBH spherulites.

Fig. 4 Spherulite (crystal) growth morphology observed by POM and crystallization half-time measured by DSC of various-MW PHBHs on the isothermal crystallization process. (A) Crystallization half-time. (B) Polarization optical microscopy images of (a) UHMW-PHBH and various MW-PHBHs [(b), (c), (d)] isothermally crystallized at 70 °C. (C) Spherulite growth rates of UHMW-PHBH and various MW-PHBHs.

Table 3 Half-time of crystallization of UHMW-PHBH and various MW-PHBHs.

Sample ($M_w \times 10^{-4}$)	$t_{1/2}$ [min]						
	T_c [°C]						
	40	50	60	70	80	90	100
400	6.6	3	2.3	1.7	2.4	4.2	7.1
140	4	2.5	1.9	1.8	3.6	4.3	20.2
99	3.6	1.9	1.4	1.5	2.3	4.7	15.4
46	4.8	2.3	1.7	1.8	4.7	3.6	8.4

All POM graphics with isothermal temperature were shown in Figure 5. The number of spherulites increases with increasing M_w . This appears to be due to an increase in the crystal forming frequency by highly entangled high-molecular-weight PHBHs. On the other hand, spherulite growth rates, shown in Figure 4C (Its detail values are shown in Table 4), display a tendency to increase with decreasing molecular weight at each isothermal crystallization temperature, which are seemed the molecular chain transfer inhibitor by high molecular chains. To summarize the above results, high M_w chains lead to a decrease in the spherulite growth rate and an increase in the crystal nuclei frequency, which conduce to be no change by high molecular effect.

3.5 Thermal degradation of UHMW-PHBH and various-MW PHBHs

Thermal degradation properties are important when using PHAs as a thermoplastic material. These materials can be degraded easily by heating to temperatures over 180 °C[14, 18]. Thus, the thermal degradation of the polymers under investigation was analyzed using hot-plate and GPC measurements near their treated temperature. The UHMW-PHBH ($M_w =$

Table 4 Radial spherulite growth rates for UHMW-PHBH and various MW-PHBHs.

Sample ($M_w \times 10^{-4}$)	G [$\mu\text{m/s}$]				
	T_c [°C]				
	70	80	90	100	110
400	1.07	1.62	1.72	1.39	0.68
140	1.17	1.70	1.65	1.22	1.05
46	1.32	1.75	1.90	1.46	1.07
14	1.43	1.78	1.96	1.53	0.95

400×10^4) was heated on the hot-plate at temperatures between 170 °C and 200 °C for time periods from 0 s to 300 s. These will subsequently be called 'isothermal heated samples'. The M_w and number-average molecular weight (M_n) of the isothermal heated samples were measured by GPC. The number average degree of polymerization (P_n) was calculated from M_n and the molecular weight of the PHBH monomer ($\text{C}_6\text{H}_{10}\text{O}_2=114$). Figure 5 shows (a) M_w , (b) M_n and (c) $1/P_n$ as a function of the isothermal heating time. The M_w and M_n decrease drastically within a short time at higher isothermal temperatures such as 200 °C. This thermal degradation behavior of UHMW-PHBH can be attributed to the scission into random molecular chains by the heating process[14, 19, 20]. When the thermal degradation occurs as a random scission, P_n can be determined by a reversible reaction with second order kinetics (Eq.1).

$$\frac{1}{P_{n,t}} = K_d t + \frac{1}{P_{n,0}} \quad (\text{Eq. 1})$$

In the above equation, P_0 , K_d and t represent the initial number average molecular weight, constant of thermal degradation and isothermal degradation time,

Fig. 5 Polarizing microphotograph of PHBH spherulites ((a) ~ (d) : $T_c = 70$ °C, (e) ~ (h) : $T_c = 90$ °C, (i) ~ (l) : $T_c = 110$ °C, (a) (e) (i) : $M_w = 400 \times 10^4$, (b) (f) (j) : $M_w = 140 \times 10^4$, (c) (g) (k) : $M_w = 46 \times 10^4$, (d) (h) (l) : $M_w = 14 \times 10^4$).

Fig. 6 (a) M_w , (b) M_n and (c) P_n as a function of melting time under different melting temperatures (●: 170 °C, ■: 180 °C, ▲: 190 °C, ×: 200 °C), and Arrhenius plots for obtaining the constant K_d .

respectively. A large K_d indicates a predisposition to thermal degradation. Figure 6c is plot of $1/P_n$ vs t and the slope represents K_d , the values for which are listed in Table 5. Comparison of the K_d values of the UHMW-PHBH and various-MW PHBHs show that the K_d of UHMW-PHBH is slightly larger than that of other PHBHs. Figure 6d is an Arrhenius plot of the $\log K_d$ vs. melting temperature (treated temperature) in accordance with Eq.2:

$$K_d = A \cdot \exp(-E_a/kT) \quad (\text{Eq. 2})$$

where A , E_a , k , and T are the Arrhenius constant, activation energy of thermal degradation, Boltzmann constant and isothermal temperature, respectively.

As mentioned above, the slope of the plots in Figure 6d gives the activation energy of thermal

degradation for the respective reactions. The E_a of UHMW-PHBH is smaller than those for PHBH, UHMW-PHB and P(3HB) reported previously[14, 21, 22]. The slightly lower thermal degradability of UHMW-PHBH obtained in this study is expected to be due to the entanglement of the UHMW-PHBH units or differences in the experimental conditions. However, we note that the UHMW-PHBH retains its high molecular weight after melting at 170 °C for several tens of seconds.

3.6 Mechanical properties and highly ordered structure of cold-drawn UHMW-PHBH films

The procedure for obtaining a one-step cold drawn film of UHMW-PHBH was described earlier. In brief, the cast film was melted at 170 °C for 20 s, and then the melted film was quenched in an ice-water-bath. The film was then drawn until a drawing ratio of 8. The cold drawn film then was annealed at 80 °C for 1 h. Figure 7 and Table 6 show the mechanical properties of the one-step cold drawn UHMW-PHBH film. The tensile strength of the one-step cold-drawn film increases drastically at drawing ratios between 4 and 8, and its tensile strength was determined to be 151 MPa. A second drawing process was applied to the one-step cold-drawn film until a second drawing ratio of 2.1, and this film was again annealed at 80 °C for 1 h to make the two-step cold-drawn film. The tensile strength of the two-step cold-drawn film (at a total drawing ratio of 16.8) is further enhanced at 208 MPa. Fischer *et al.* reported the mechanical

Table 5 Thermal degradation constant: K_d (A) and activation energy: E_a (B) of UHMW-PHBH and NMW-PHBH.

A	$k_d \times 10^3$ [min ⁻¹] of each Samples		
	Treated temperature [°C]	UHMW-PHBH	PHBH ($M_w = 55 \times 10^4$) (Kabe et al.)
	170	0.036	0.018
	180	0.038	0.046
	190	0.141	0.072
	200	0.170	0.147

B	E_a [kJ/mol] of each Samples		
	UHMW-PHBH ($M_w = 55 \times 10^4$) (Kabe et al. ¹⁴)	UHMW-P(3HB) ($M_w = 370 \times 10^4$) (Aoyagi. ²¹)	P(3HB) ($M_w = 40 \times 10^4$) (Aoyagi et al. ²²)
	103	107	111

Fig. 7 Tensile strength, elongation at break, and Young's modulus of one- and two-step cold-drawn UHMW-PHBH films.

properties of cold-drawn PHBH films prepared by a similar method using P(3HB-co-5 mol% -3HH) with a NMW ($M_w=82 \times 10^4$), and the tensile strength of a film at a total drawing ratio of 10 was shown to be 140 MPa[11]. This comparison can be used as a guide to determine the effect of ultra-high molecular weight on the mechanical properties of PHBH. To our knowledge, the value of 208 MPa obtained in this study is the highest tensile strength reported for a

drawn film of PHBH. Comparing two-step cold-drawn films at the total drawing ratio of 12.8 and 16.8, tensile strength remained unchanged. However, Young's modulus decreased with increasing second drawing ratio. Since total drawing ratio at 16.8 seems to draw film too much, the structure formed by the one-step cold-drawing process might be broken.

WAXD patterns were obtained to determine the factors giving rise to the increased tensile strength. Figure 8 shows the WAXD patterns of the one- and two-step cold-drawn UHMW-PHBH films. Table 7 lists the parameters calculated from the WAXD and SAXS patterns. The WAXD pattern of the undrawn film (Figure 8a) shows a less orientated diffraction pattern arising from the α -form crystal of P(3HB) [23, 24]. In the Figures 8b and c, the WAXD patterns of the one-step cold-drawn UHMW-PHBH films at drawing ratios of 4 and 8 show reflections from the β -form structure of P(3HB), named as ' β -form diffraction', with strong reflections such as $\alpha(020)$ and $\alpha(110)$ on the equatorial line. The β -form diffractions appear to be related to the increased tensile strength of the PHA films, since the fibers giving rise to the β -form diffraction appeared in the drawn PHA films or fiber with high tensile strength. Until now, β -form diffraction has mostly been observed in two-step drawn PHA films, with only a few exceptions[16, 25]. Therefore, the β -form structure appears to be generated from tie-chains, which exist between lamellar crystals, by secondly drawing process.

In a previous study, we investigated the mechanical properties and highly ordered structure of drawn blend films composed of UHMW-P(3HB) and NMW P(3HB)[16]. While one-step cold-drawn pure UHMW-P(3HB) films did not contain β -form structures, one-step cold-drawn blend films composed of equal parts UHMW-P(3HB) and NMW-P(3HB) exhibited the β -form, similar to the case of one-step cold-drawn UHMW-PHBH reported in this paper. In the case of the UHMW-P(3HB), the drawing ratio was

Table 6 Tensile strength, elongation at break and Young's modulus of one- and two-step cold drawn UHMW-PHBH films.

1st DR	2nd DR	TDR	Tensile strength [MPa]	Elongation at break [%]	Young's modulus [GPa]
1.0	-	1.0	13 $\pm$ 8	4 $\pm$ 2	1.17 $\pm$ 0.21
4.0	-	4.0	29 $\pm$ 14	150 $\pm$ 58	1.14 $\pm$ 0.23
	-	8.0	151 $\pm$ 63	87 $\pm$ 38	1.29 $\pm$ 0.32
8.0	1.6	12.8	208 $\pm$ 26	42 $\pm$ 1	3.45 $\pm$ 0.30
	2.1	16.8*	207	38	1.48

*The number of tests for this data is one, because this film with the drawing ratio of 16.8 was very difficult to preparation.

Fig. 8 WAXD diagrams of one- and two-step cold-drawn UHMW-PHBH films.

Table 7 Crystallinity, long period, lamellar crystal thickness, orientation, and $R_{\beta/\alpha}$ values of one- and two-step cold-drawn UHMW-PHBH films.

TDR	X_c [%]	L [nm]	l_c [nm]	F [%]	$R_{\beta/\alpha(020)}$
1.0	76.3	6.62	5.1	73.5	0
4.0	72.8	6.61	4.8	94.8	0.19
8.0	70.3	7.70	5.4	93.6	0.24
12.8 (8.0 × 1.6)	81.5	8.78	7.2	95.7	0.45
16.8 (8.0 × 2.1)	75.7	9.79	7.4	95.4	0.47

found to be lower than that of NMW-P(3HB) owing to the higher entanglement of the latter. In addition, UHMW-P(3HB) chains in the blend film are located across some of the lamellar crystals as tie-chains. Due to this arrangement, the β -form structure can be generated from the tie-chains with a thickening of the lamellar crystals by an annealing process. The forming of β -form structures in one-step cold-drawn UHMW-PHBH films seems to be due to the lower entanglement of UHMW-PHBH compared to that of UHMW-P(3HB), mainly arising from large side chains on the 3HH unit. In other words, the drawing of a structure that has many tie-chain and appropriate entanglement leads to the formation of the β -form structure.

When comparing the one-step cold-drawn UHMW-PHBH film with a drawing ratio 8 and the two-step cold-drawn UHMW-PHBH film with a drawing ratio 12.8, the β -form diffraction of the two-step cold-drawn film was higher, as seen in Figures 8c and d. In order to estimate the amount of the β -form structure, the intensity ratio ($R_{\beta/\alpha}$) was calculated using the following equation;

$$R_{(\beta/\alpha)} = \int I_{\beta} dI / \int I_{\alpha(020)} dI \quad (\text{Eq.3})$$

$\int I_{\beta} dI$ and $\int I_{\alpha(020)} dI$ were calculated by integrating the β -diffraction and the $\alpha(020)$ diffraction, respectively. The crystallinity, lamellar long period (L), lamellar thickness (l_c), orientation (F), and intensity ratio ($R_{\beta/\alpha}$) are listed in Table 4. It is found that L , l_c and $R_{\beta/\alpha}$ increase significantly between one- and two-step

cold-drawn processes. Base on the above results, the second drawing process seems to lead to the formation of the β -form structure from tie-chains that existed between stacked lamellar crystals, which were formed by the first drawing and annealing process.

4. Conclusion

In this paper, we introduce an optimal extraction method for the ultra-high-molecular-weight poly[(*R*)-3-hydroxybutyrate - *co* - (*R*) - 3 - hydroxyhexanoate] (UHMW-PHBH) microbial polyester. In addition, various-MW PHBHs with wide range molecular weights from 1×10^4 to 400×10^4 were prepared from UHMW-PHBH by H_2SO_4 treatment.

The effect of molecular weight on the thermal properties, crystal morphology and mechanical properties were investigated with the aid of these samples. Based on DSC measurement results, it can be concluded that a molecular weight over 62×10^4 does not affect the T_m , T_c and T_g . Observation of spherulite growth morphology provides evidence that high-molecular-weight chains lead to a decrease in the spherulite growth rate and an increase in crystal forming frequency, which conduce to be no change because the two effects cancel out. The stability of the UHMW-PHBH to thermal degradation was slightly decreased compared to NMW PHBH, which appears to be due to the increasing entanglement. Furthermore, the results presented here represent the first estimate of the mechanical properties of

UHMW-PHBH.

One-step cold-drawn UHMW-PHBH films were observed to have the β -form structure, despite the use of only one cold drawing step. This is the result of the inclusion of many tie-chains of PHBH within the polymer matrix. Two-step cold-drawing method proved effective in improving the mechanical properties of the UHMW-PHBH films. The additional improving for mechanical properties of UHMW-PHBH require to prevent its molecular entanglement.

Aknowledgment

This work was partially supported by a Grant-in-Aid for Scientific Research of Japan (A) (No. 26248044 (2014)); the CREST program of the Japan Science and Technology Agency (JST) and Photon and Quantum Basic Research Coordinated Development Program of MEXT. The synchrotron radiation experiments were performed at SPring-8 with the approval of RIKEN (Proposal Nos. 20150080 and 20160020) at the BL45XU or Frontier Softmaterial Beamline (Proposal Nos. 2016 A1861 and 2016B1966) at BL03XU, and advice and help given by Dr Hiroyasu Masunaga has been a great help in some synchrotron radiation experiments in BL03XU.

References

1. L. L. Madison, G. W. Huisman, *Microbiol Mol Biol Rev*, **63**, 21–53 (1999).
2. K. Sudesh, H. Abe, Y. Doi, *Prog Polym Sci*, **25**, 1503–55 (2000).
3. R. W. Lenz, R. H. Marchessault, *Biomacromolecules*, **6**, 1–8 (2005).
4. P. A. Holmes, *Physics in Technology*, **16**, 32–6 (1985).
5. M. Liebergesell, F. Mayer, A. Steinbuchel, *Appl Microbiol Biotechnol*, **40**, 292–300 (1993).
6. M. Kunioka, Y. Nakamura, Y. Doi, *Polymer Communications*, **29**, 174–6 (1988).
7. N. Tanadchangsang, A. Kitagawa, T. Yamamoto, H. Abe, T. Tsuge, *Biomacromolecules*, **10**, 2866–74 (2009).
8. F. Biddlestone, A. Harris, J. N. Hay, T. Hammond, *Polym Int*, **39**, 221–9 (1996).
9. G. J. M. Dekoning, P. J. Lemstra, *Polymer*, **33**, 3292–4 (1992).
10. T. Iwata, K. Tsunoda, Y. Aoyagi, S. Kusaka, N. Yonezawa, Y. Doi, *Polym Degrad Stab*, **79**, 217–24 (2003).
11. J. J. Fischer, Y. Aoyagi, M. Enoki, Y. Doi, T. Iwata, *Polym Degrad Stab*, **83**, 453–60 (2004).
12. T. Iwata, M. Fujita, Y. Aoyagi, Y. Doi, T. Fujisawa, *Biomacromolecules*, **6**, 1803–9 (2005).
13. K. J. Kim, Y. Doi, H. Abe, *Polym Degrad Stab*, **91**, 769–77 (2006).
14. T. Kabe, C. Hongo, T. Tanaka, T. Hikima, M. Takata, T. Iwata, *J Appl Polym Sci*, **132**, 41258 (2015).
15. T. G. Fox, P. J. Flory, *J Appl Phys*, **21**, 581–91 (1950).
16. T. Kabe, T. Tsuge, K. Kasuya, A. Takemura, T. Hikima, M. Takata, et al., *Macromolecules*, **45**, 1858–65 (2012).
17. H. Alata, T. Aoyama, Y. Inoue, *Macromolecules*, **40**, 4546–51 (2007).
18. T. Kabe, T. Tsuge, T. Hikima, M. Takata, A. Takemura, T. Iwata, *ACS Symposium series*, **1105**, 63–75 (2012).
19. N. Grassie, E. J. Murray, P. A. Holmes, *Polym Degrad Stab*, **6**, 95–103 (1984).
20. M. Kunioka, Y. Doi, *Macromolecules*, **23**, 1933–6 (1990).
21. Y. Aoyagi, Saitama University (2003).
22. Y. Aoyagi, K. Yamashita, Y. Doi, *Polym Degrad Stab*, **76**, 53–9 (2002).
23. K. Okamura, R. H. Marchessault, *Conformation of Biopolymers*. London: Academic Press, p. 709 (1967).
24. M. Yokouchi, Y. Chatani, H. Tadokoro, K. Teranish, H. Tani, *Polymer*, **14**, 267–72 (1973).
25. J. Q. Zhang, K. Kasuya, T. Hikima, M. Takata, A. Takemura, T. Iwata, *Polym Degrad Stab*, **96**, 2130–8 (2011).