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1 Biosynthesis of poly(glycolate-co-3-hydroxybutyrate-co-3-  
2 hydroxyhexanoate) in *Escherichia coli* expressing sequence-  
3 regulating polyhydroxyalkanoate synthase and medium-chain-  
4 length 3-hydroxyalkanoic acid coenzyme A ligase

5

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18

19 Running title: A glycolate-based polyhydroxyalkanoate terpolymer

20

21 Keywords: polyhydroxyalkanoate, block copolymer, glycolate, 3-hydroxyhexanoate

22

23 **Abstract**

24 Chimeric polyhydroxyalkanoate synthase Pha<sub>CAR</sub> is characterized by the  
25 capacity to incorporate unusual glycolate (GL) units and spontaneously synthesize block  
26 copolymers. The GL and 3-hydroxybutyrate (3HB) copolymer synthesized by Pha<sub>CAR</sub> is  
27 a random-homo block copolymer, poly(GL-*ran*-3HB)-*b*-poly(3HB). In the present study,  
28 medium-chain-length 3-hydroxyhexanoate (3HHx) units were incorporated into this  
29 copolymer using Pha<sub>CAR</sub> for the first time. The coenzyme A (CoA) ligase from  
30 *Pseudomonas oleovorans* (AlkK) serves as a simple 3HHx-CoA supplying route in  
31 *Escherichia coli* from exogenously supplemented 3HHx. NMR analyses of the obtained  
32 polymers revealed that 3HHx units were randomly connected to 3HB units, whereas GL  
33 units were heterogeneously distributed. Therefore, the polymer is composed of two  
34 segments: P(3HB-*co*-3HHx) and P(GL-*co*-3HB-*co*-3HHx). The thermal and mechanical  
35 properties of the terpolymer indicate no contiguous P(3HB) segments in the material,  
36 consistent with the NMR results. Therefore, Pha<sub>CAR</sub> synthesized the novel block  
37 copolymer P(3HB-*co*-3HHx)-*b*-P(GL-*co*-3HB-*co*-3HHx), which is the first block PHA  
38 copolymer comprising two copolymer segments.

39

40

41 Polyhydroxyalkanoates (PHAs) are bacterial storage polyesters that can be  
42 produced from various renewable biomass and used as plastic materials (Zhang *et al.*  
43 2018; Bedade *et al.* 2021). PHA synthase (PhaC) plays a central role in the biosynthetic  
44 pathway of PHAs (Rehm 2003). PhaC is typically specific to 3-hydroxyacyl-coenzyme A  
45 (CoA) substrates (Steinbüchel and Hein 2001; Sudesh *et al.* 2000) and synthesizes  
46 homopolymers and/or random copolymers, which have no regulated monomer sequence,  
47 with multiple monomer substrates (Doi *et al.* 1995; Bartels *et al.* 2020).

48 Sequence-regulating PhaC is a recently discovered type of enzyme which is

49 capable of spontaneously synthesizing block copolymers without the manipulation of  
50 feedstocks during cultivation (Matsumoto *et al.* 2018). PhaC<sub>AR</sub>, which is a unique  
51 sequence-regulating PHA synthase, is an engineered chimeric enzyme composed of PHA  
52 synthases from *Aeromonas caviae* and *Ralstonia eutropha* (*Cupriavidus necator*)  
53 (Matsumoto *et al.* 2009). Notably, PhaC<sub>AR</sub> possesses unusual substrate specificity toward  
54 glycolyl (GL)-CoA (Arai *et al.* 2020). We previously reported that PhaC<sub>AR</sub> synthesized a  
55 block copolymer of GL and 3-hydroxybutyrate (3HB), poly(GL-*ran*-3HB)-*b*-poly(3HB).

56 GL-based polymers are characterized by their high hydrolytic degradability. For  
57 example, chemically synthesized polyglycolic acid (PGA) and poly(lactide-*co*-glycolide)  
58 (PLGA) can be hydrolyzed without the action of esterases (Pandita, Kumar and Lather  
59 2015). Such hydrolytically degradable polymers have the potential to undergo hydrolysis  
60 in animal tissues; thus, they have the potential to serve as rapidly bioabsorbable materials  
61 (Pervaiz *et al.* 2019; Shawe *et al.* 2006). In contrast, natural PHAs have low hydrolytic  
62 degradability and slow bioabsorption (Basnett *et al.* 2018; Chen and Zhang 2018). GL-  
63 based PHAs potentially exhibit an intermediate hydrolytic degradability between  
64 chemically prepared GL-based polymers and natural PHAs. In fact, the first GL-based  
65 PHA random copolymer P(GL-*co*-3HB) synthesized using PhaC<sub>1Ps</sub>STQK hydrolyzes in  
66 the absence of PHA depolymerases (Matsumoto *et al.* 2011; Matsumoto *et al.* 2017).

67 P(GL-*ran*-3HB)-*b*-P(3HB) synthesized by PhaC<sub>AR</sub> contains 23 mol% GL, which  
68 is higher than that of PhaC<sub>1Ps</sub>STQK (16 mol%) obtained under the same culture  
69 conditions (Arai 2020). The block copolymer contains a GL-rich segment with a local GL  
70 fraction of 71 mol%, indicating the superior GL-incorporating capacity of PhaC<sub>AR</sub>.  
71 However, a drawback of P(GL-*ran*-3HB)-*b*-P(3HB) is the stiff and brittle properties of  
72 the produced material. A potentially effective strategy to improve the material properties  
73 of PHA is the incorporation of 3-hydroxyhexanoate (3HHx) units into the polymer. A  
74 successful example is P(3HB-*co*-3HHx), also known as PHBH (Sato *et al.* 2015), which

75 exhibits higher flexibility than P(3HB) (Wong *et al.* 2012). Here, we report the  
76 incorporation of 3HHx as the third monomer into the copolymer with GL and 3HB  
77 repeating units by Pha<sub>CAR</sub> to improve the physical properties. Analysis of the structure  
78 and physical properties of the obtained polymer indicated that they are representative of  
79 a new GL-based block terpolymer with transparent and superior extensible properties.

80 To supply 3HHx-CoA, 3-hydroxyacyl-CoA ligase from *Pseudomonas*  
81 *oleovorans* (AlkK) (Wang *et al.* 2012) was utilized. In PHA-producing *Pseudomonas*  
82 strains, the enzyme serves as a *de novo* medium-chain-length (MCL) monomer-supplying  
83 pathway together with the 3-hydroxyacyl-acyl carrier protein thioesterase (PhaG) (Rehm,  
84 Krüger and Steinbüchel 1998; Wang 2012). The *alkK* gene is functionally expressed in  
85 *Escherichia coli* (Satoh *et al.* 2005). Heterologous expression of PhaG and AlkK in *E.*  
86 *coli* enables the synthesis of MCL PHA from non-fatty acid carbon sources (Scheel *et al.*  
87 2019; Tappel *et al.* 2014). In the present study, we utilized AlkK to supply 3HHx-CoA  
88 from exogenous 3HHx supplemented into the medium. This enzyme serves as an easy-  
89 to-use 3HHx-CoA supplying route in *E. coli*.

90

91

## 92 **Materials and Methods**

### 93 **Plasmid construction for polymer production.**

94 A plasmid for synthesizing P(GL-*co*-3HB-*co*-3HHx) was constructed based on  
95 pBSP<sub>RephaCAR</sub>pct (Matsumoto *et al.* 2018). An *Af*III recognition site was introduced  
96 downstream of the *pct* gene. Using this site, *alkK* from *P. oleovorans*, which encodes acyl-  
97 CoA ligase, was introduced. The resulting plasmid pBSP<sub>RephaCAR</sub>pctalkK was confirmed  
98 not to possess any unintended mutations.

99

### 100 **Preparation of 3HHx**

101 Ethyl (*R,S*)-3-hydroxyhexanoate was hydrolyzed by adding an excess amount of  
102 10 N NaOH on ice until the solution was no longer phase separated. The solution was  
103 acidified to approximately pH 2 by adding 6 N HCl, and diethylether was then added to  
104 extract 3-hydroxyhexanoic acid. Diethylether was removed *in vacuo*, and the residues  
105 were dissolved in water and neutralized by NaOH to give sodium 3HHx (3HHx-Na)  
106 solution.

107

### 108 **Polymer production and analysis**

109 *E. coli* JM109 was used as the host for plasmid construction and polymer  
110 production. *E. coli* was cultivated in 1.5 mL Luria Bertani medium (10 g L<sup>-1</sup> NaCl, 5 g L<sup>-1</sup>  
111 yeast extract, and 10 g L<sup>-1</sup> tryptone) containing ampicillin (100 mg L<sup>-1</sup>) at 30°C for 12  
112 h as the seed culture. For polymer production, cells harboring the plasmid for polymer  
113 production were cultivated at 30°C for 48 h. As monomer precursors, 3HB-Na, GL-Na,  
114 and 3HHx-Na were added at 1.25 or 2.5 g L<sup>-1</sup>, 2.5 or 5.0 g L<sup>-1</sup>, and 0-2.5 g L<sup>-1</sup>, respectively.  
115 The polymer content and monomer composition were analyzed by gas chromatography  
116 as described previously (Taguchi *et al.* 2008). The polymer used for subsequent analysis  
117 was extracted from cells at 12 or 24 h cultivation. The intracellular polymer in lyophilized  
118 cells was extracted using chloroform at 60°C for 48 h.

119 <sup>1</sup>H NMR and <sup>13</sup>C NMR analyses of the extracted polymer in CDCl<sub>3</sub> were carried  
120 out as described previously (Arai 2020). The molecular weight of the polymer was  
121 measured by size exclusion chromatography using polystyrene standards for calibration,  
122 as described previously (Arai 2020).

123

### 124 **Preparation of solvent-cast films and mechanical properties analysis**

125 Solvent-cast films of the purified polymers were prepared as follows:  
126 Approximately 400 mg of purified polymer was dissolved in 10 mL of chloroform. The

127 solution was then placed in a glass Petri dish, which was covered with aluminum foil with  
128 10 holes ( $\phi \sim 1$  mm), and placed on a horizontal table at room temperature to allow the  
129 solution to evaporate. After 2 weeks, the obtained circular film was further dried *in vacuo*  
130 for 24 h to remove any residual solvent. The resulting films were stored at room  
131 temperature for at least 2 more weeks prior to testing.

132 The tensile strength, Young's modulus, and elongation to break of the films were  
133 determined using a tensile testing machine (EZ-test, Shimadzu, Japan) operated at a  
134 tensile speed of 3 mm/min at room temperature. Samples were cut from the films using a  
135 dumbbell-shaped cutter SDMP-1000-D (Dumbbell, Japan), with a gauge length and width  
136 of 12 mm and 2 mm, respectively.

137

### 138 **Thermal properties analysis**

139 The glass transition temperature ( $T_g$ ) and melting temperature ( $T_m$ ) of the  
140 synthesized polymers were analyzed by differential scanning calorimetry (DSC) analysis  
141 using DSC-8500 (PerkinElmer). Approximately 5-10 mg of each polymer was confined  
142 in an aluminum pan using a pressing machine (Mettler Toledo). Measurement was  
143 performed under nitrogen atmosphere (flow rate: 100 ml/min) at the following  
144 temperature control: (1) cooling from 30°C to -30°C at 50°C/min, (2) cooling from -30°C  
145 to -50°C at 20°C/min, (3) heating from -50°C to 210°C at 20°C/min, (4) cooling from  
146 210°C to -30°C at 50°C/min, (5) cooling from -30°C to -50°C at 20°C/min, (6) isothermal  
147 heating at -50°C for 5 min, and (7) heating from -50°C to 210°C at 20°C/min.

148

149

## 150 **Results and Discussion**

### 151 **Biosynthesis of GL-based PHAs containing 3HHx units**

152 The metabolic pathway used to synthesize PHAs containing GL, 3HB, and

153 3HHx is shown in Fig. 1. GL, 3HB, and 3HHx were supplemented to the medium and  
154 taken up by *E. coli* cells. Propionyl-CoA transferase (PCT) derived from *Megasphaera*  
155 *elsdenii* converts GL and 3HB to GL-CoA and 3HB-CoA, respectively, using acetyl-CoA  
156 as a CoA donor. Acyl-CoA ligase (AlkK) activates 3HHx using ATP and free CoA to  
157 produce 3HHx-CoA (Wang 2012).

158 First, we carried out polymer production with various concentrations of 3HHx-  
159 Na and fixed concentrations of GL-Na and 3HB-Na (Table 1). In this way, we successfully  
160 incorporated 3HHx into the copolymers. The supplemented 3HHx-Na was increased to 2  
161 g L<sup>-1</sup>, resulting in a subsequent increase in the 3HHx content but a decrease in the relative  
162 ratio of GL content within the copolymer (Table 1, No. 1-4). The 3HB fraction  
163 incorporated into the copolymers remained nearly constant despite the changing ratios of  
164 the other feedstocks. On the other hand, the cell dry weight (CDW) decreased as the  
165 3HHx-Na concentration increased, particularly at concentrations above 2 g L<sup>-1</sup>,  
166 suggesting that 3HHx inhibits cell growth (No. 5). In contrast, no significant changes in  
167 PHA production were observed. When we attempted polymer production in the absence  
168 of AlkK, the incorporation of 3HHx decreased compared to that in the presence of AlkK  
169 (Table S1). This result clearly demonstrates that AlkK plays an important role in  
170 providing 3HHx-CoA for polymer production.

171

## 172 **Sequence analysis of 3HHx units**

173 Due to the sequence-regulating capacity of PhaC<sub>AR</sub>, the monomer sequence of  
174 the obtained polymer was of interest. Thus, the monomer sequence was analyzed based  
175 on changes in NMR chemical shifts influenced by the chemical structures of the adjacent  
176 units. The linkages between the 3HB and 3HHx units were determined by the <sup>13</sup>C NMR  
177 resonances of carbonyl carbons. As shown in Fig. 2 and Fig. S1, three signals, which are  
178 ascribed to the carbonyl group of 3HB and 3HHx, were observed at δ 169.0-170.0. These

179 three signals correspond to the dyad sequences of 3HHx\*-3HHx, 3HB\*-3HHx or 3HHx\*-  
180 3HB, and 3HB\*-3HB, respectively (Phithakrotchanakoon *et al.* 2015; Shimamura *et al.*  
181 1994). Here, the asterisk indicates the focused unit of which the signal was observed. The  
182 abundant 3HB-3HHx/3HHx-3HB linkages indicate that 3HHx units were randomly  
183 incorporated into the polymer chain.

184 The resonances of the carbonyl carbon of the GL units were observed as split peaks  
185 in the range of  $\delta$  165.0-167.0 due to the triad sequence including GL units (Matsumoto  
186 2011; Matsumoto 2017). However, for the spectrum for polymer **2** which contains 17  
187 mol% GL, the resonance of GL units was not clearly observed due to the low signal  
188 intensity. In addition, as GL has a short main-chain, its resonance can be subject to be  
189 influenced by the adjacent monomer units. The signal of the carbonyl carbon of GL is  
190 predicted to be divided into nine triad patterns (3HB/3HHx/GL-GL\*-3HB/3HHx/GL),  
191 therefore the intensity could be very weak to be detected. Indeed, for another terpolymer  
192 harboring 19 mol% GL, the signals were slightly observed (Fig. S2).

193

#### 194 **Sequence analysis of GL units**

195 Previous research has demonstrated that the <sup>1</sup>H NMR resonance of the methylene  
196 proton of GL in P(GL-*ran*-3HB) is observed as four characteristic signals at 4.5-4.9 ppm,  
197 which are ascribed to GL-GL\*-GL (a), GL-GL\*-3HB or 3HB-GL\*-GL [(b) or (c)], and  
198 3HB-GL\*-3HB (d) triad sequences, respectively (Fig. 3) (Arai 2020; Matsumoto 2017).  
199 Similarly, the terpolymer exhibits four similar signals that correspond to GL-GL\*-GL (a),  
200 GL-GL\*-(3HB/3HHx) or (3HB/3HHx)-GL\*-GL [(b) or (c)], and (3HB/3HHx)-GL\*-  
201 (3HB/3HHx) (d), respectively (Fig. S3). The effects of 3HB and 3HHx units in the triads  
202 on the chemical shift of the GL proton were indistinguishable. Based on the relative  
203 intensities of the four signals, the local GL fraction, which is defined as the molar ratio of  
204 GL units in a segment of P(GL-*co*-3HB) (Arai 2020), can be calculated (Table 1, Table

205 S2). In our previous study, for example, we found that P(40 mol% GL-*co*-3HB) has a GL-  
206 rich segment in which the local GL fraction is estimated to be 71 mol% (Arai 2020). Here,  
207 we calculated the local GL fraction of the terpolymer in the same way (Table 1, Table S2).  
208 In all of the polymers produced and analyzed, the local GL fractions were higher than the  
209 total GL fractions, clearly indicating the heterologous distribution of GL units in the  
210 polymer chain. For polymer **1** P(GL-*co*-3HB), the resonance pattern was similar to that  
211 in our previous study (Arai 2020). The intensity of (a) (**a** in Table S2) was the highest  
212 among the four signals, followed by **b** and **c**, and **d** was the lowest (Table S2). This result  
213 demonstrates that the GL-rich segment is present in polymer **1**. For polymers **2-4**, as the  
214 3HHx fraction increased, **a-c** decreased whereas **d** increased. The local GL fraction of  
215 polymer **2** was 56 mol% (Table 1). The ratio of the GL-rich segment in **1** was estimated  
216 to be 39%, which is higher than that in **2** and **3** (30%), whereas the ratio and the local GL  
217 fraction of **4** could not be determined due to its low signal intensity of GL units. Therefore,  
218 the ratio of the GL-rich segment was reduced by the incorporation of 3HHx.

219         Based on the results of NMR analysis, we propose the polymer structures (Fig.  
220 4). The structure for polymer **1** is similar to that observed in our previous study, which is  
221 P(GL-*ran*-3HB)-*b*-P(3HB) (Arai 2020). For polymer **2**, the <sup>13</sup>C NMR results suggest the  
222 presence of a P(3HB-*co*-3HHx) segment while <sup>1</sup>H NMR suggests the presence of a  
223 terpolymer segment. Thus, **2** can be presumed to be P(3HB-*co*-3HHx)-*b*-P(GL-*co*-3HB-  
224 *co*-3HHx). The monomer sequence in each segment is presumably random, although the  
225 statistical randomness in the terpolymer segment cannot be determined from the obtained  
226 data. For polymer **4**, the GL-rich segment disappeared; thus, it is presumed to be P(GL-  
227 *co*-3HB-*co*-3HHx).

228         It should be noted that the current analysis does not determine the number and  
229 the order of the segments. Fig. 4 depicted the total amount (ratio) of each segment, but  
230 does not necessarily mean that the obtain polymer is a diblock copolymer.

231

### 232 **Mechanical properties of solvent-cast films**

233           The solvent-cast film of P(20 mol% GL-*co*-3HB) (Arai 2020) was opaque due  
234 to the high crystallinity of P(3HB), whereas those of polymers **2** and **3** were transparent  
235 (Fig. 5). These results suggest that the incorporation of 3HHx lowers the crystallinity of  
236 the polymers. Next, stress-strain tests were carried out on the films at room temperature  
237 (Fig. S4 and Table 2). The Young's modulus of polymers **2** and **3** [P(GL-*co*-3HB-*co*-  
238 3HHx)] was much lower than that of **1** [P(GL-*co*-3HB)] and PHBH, indicating that the  
239 terpolymer is a very soft and extensible material.

240

### 241 **Thermal properties of the polymers**

242           The thermal properties of the synthesized polymers were determined (Fig. 6 and  
243 Table 3). Polymer **1** exhibited a melting peak at 157.6°C, which can be ascribed to the  
244 relatively large crystalline fraction of P(3HB). For the polymers containing 3HHx, as the  
245 3HHx fraction increased, the area of the melting peak decreased and eventually  
246 disappeared. This result indicates that the incorporation of 3HHx units drastically lowered  
247 the crystallinity of the polymer, and that the P(3HB) homopolymer segment does not exist  
248 in polymers **2-4**. This finding is consistent with appearance and physical properties of the  
249 solvent-cast films described above and further supports the proposed structures shown in  
250 Fig. 4.

251

252

### 253 **Conclusions**

254           The sequence-regulating PHA synthase PhaC<sub>AR</sub> synthesized a novel GL-based  
255 terpolymer P(3HB-*co*-3HHx)-*b*-P(GL-*co*-3HB-*co*-3HHx), which is the first PHA block  
256 copolymer composed of two random copolymer segments. An inverse relationship was

257 observed between GL and 3HHx fractions. In addition, the ratio of GL-containing  
258 segments was reduced by introducing 3HHx units. However, the mechanism of this  
259 phenomenon at the molecular level remains unclear. To achieve the combination of  
260 hydrolytic degradability and flexible properties, a greater ratio of GL-rich segments  
261 containing 3HHx units is preferable. Therefore, further improvement of the biosynthetic  
262 system is needed. The hydrolytic degradability and bioabsorption of the terpolymer and  
263 P(GL-*co*-3HB) synthesized using PhaC<sub>1Ps</sub>STQK will be addressed in our future work.

264

265

#### 266 **Authors' contributions**

267 H.T. analyzed data and wrote the manuscript. K.S. performed the experiments, analyzed  
268 data, and wrote the manuscript. C.N. provided the *alkK* gene and wrote the manuscript.  
269 K.M. designed and supervised this study, analyzed data, and wrote the manuscript. All  
270 authors read and approved the final version of the manuscript.

271

272

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276

#### 277 **Conflict of Interest**

278 The authors declare no conflict of interest.

279

#### 280 **Data Availability Statement**

281 The data underlying this article are available in the article and in its online supplementary  
282 material.

283

284 **References**

285 Arai S, Sakakibara S, Mareschal R et al. Biosynthesis of random-homo block copolymer  
286 poly[glycolate-*ran*-3-hydroxybutyrate (3HB)]-*b*-Poly(3HB) using sequence-regulating  
287 chimeric polyhydroxyalkanoate synthase in *Escherichia coli*. *Front Bioeng Biotechnol*  
288 2020;8:612991.

289

290 Bedade DK, Edson CB, and Gross RA. Emergent approaches to efficient and sustainable  
291 polyhydroxyalkanoate production. *Molecules* 2021;26:3463.

292

293 Bartels M, Gutschmann B, Widmer T et al. Recovery of the PHA copolymer P(HB-*co*-  
294 HHx) with non-halogenated solvents: influences on molecular weight and HHx-content.  
295 *Front Bioeng Biotechnol* 2020;8:944.

296

297 Basnett P, Marcello E, Lukaszewicz B et al. Biosynthesis and characterization of a novel,  
298 biocompatible medium chain length polyhydroxyalkanoate by *Pseudomonas mendocina*  
299 CH50 using coconut oil as the carbon source. *J Mater Sci Mater Med* 2018;29:179.

300

301 Chen GQ, Zhang J. Microbial polyhydroxyalkanoates as medical implant biomaterials.  
302 *Artif Cells Nanomed Biotechnol* 2018;46:1-18.

303

304 Doi Y, Kitamura S, and Abe H. Microbial synthesis and characterization of poly(3-  
305 hydroxybutyrate-*co*-3-hydroxyhexanoate). *Macromolecules* 1995;28:4822-4828.

306

307 Matsumoto K, Hori C, Fujii R et al. Dynamic changes of intracellular monomer levels  
308 regulate block sequence of polyhydroxyalkanoates in engineered *Escherichia coli*.

309 *Biomacromolecules* 2018;19:662-71.

310

311 Matsumoto K, Ishiyama A, Sakai K et al. Biosynthesis of glycolate-based polyesters  
312 containing medium-chain-length 3-hydroxyalkanoates in recombinant *Escherichia coli*  
313 expressing engineered polyhydroxyalkanoate synthase. *J Biotechnol* 2011;156:214-7.

314

315 Matsumoto K, Shiba T, Hiraide Y et al. Incorporation of glycolate units promotes  
316 hydrolytic degradation in flexible poly(glycolate-co-3-hydroxybutyrate) synthesized by  
317 engineered *Escherichia coli*. *ACS Biomater Sci Eng* 2017;3:3058-63.

318

319 Matsumoto K, Takase K, Yamamoto Y et al. Chimeric enzyme composed of  
320 polyhydroxyalkanoate (PHA) synthases from *Ralstonia eutropha* and *Aeromonas caviae*  
321 enhances production of PHAs in recombinant *Escherichia coli*. *Biomacromolecules*  
322 2009;10:682-5.

323

324 Pandita D, Kumar S, Lather V. Hybrid poly(lactic-co-glycolic acid) nanoparticles: design  
325 and delivery prospectives. *Drug Discov Today* 2015;20:95-104.

326

327 Pervaiz F, Ahmad M, Li L et al. Development and characterization of olanzapine loaded  
328 poly(lactide-co-glycolide) microspheres for depot injection: *in vitro* and *in vivo* release  
329 profiles. *Curr Drug Deliv* 2019;16:375-83.

330

331 Phithakrotchanakoon C, Champreda V, Aiba S-I et al. Production of  
332 polyhydroxyalkanoates from crude glycerol using recombinant *Escherichia coli*. *J Polym*  
333 *Environ* 2015;23:38-44.

334

335 Rehm BH. Polyester synthases: natural catalysts for plastics. *Biochem J* 2003;376:15-33.  
336

337 Rehm BH, Krüger N, Steinbüchel A. A new metabolic link between fatty acid *de novo*  
338 synthesis and polyhydroxyalkanoic acid synthesis. The PHAG gene from *Pseudomonas*  
339 *putida* KT2440 encodes a 3-hydroxyacyl-acyl carrier protein-coenzyme a transferase. *J*  
340 *Biol Chem* 1998;273:24044-51.  
341

342 Sato S, Maruyama H, Fujiki T et al. Regulation of 3-hydroxyhexanoate composition in  
343 PHBH synthesized by recombinant *Cupriavidus necator* H16 from plant oil by using  
344 butyrate as a co-substrate. *J Biosci Bioeng* 2015;120:246-51.  
345

346 Satoh Y, Murakami F, Tajima K et al. Enzymatic synthesis of poly(3-hydroxybutyrate-*co*-  
347 4-hydroxybutyrate) with CoA recycling using polyhydroxyalkanoate synthase and acyl-  
348 CoA synthetase. *J Biosci Bioeng* 2005;99:508-11.  
349

350 Scheel RA, Fusi AD, Min BC et al. Increased production of the value-added biopolymers  
351 poly(*R*-3-hydroxyalkanoate) and Poly( $\gamma$ -glutamic acid) from hydrolyzed paper recycling  
352 waste fines. *Front Bioeng Biotechnol* 2019;7:409.  
353

354 Shawe S, Buchanan F, Harkin-Jones E et al. A study on the rate of degradation of the  
355 bioabsorbable polymer polyglycolic acid (PGA). *J Mater Sci* 2006;41:4832-38.  
356

357 Shimamura E, Kasuya K, Kobayashi G et al. Physical properties and biodegradability of  
358 microbial poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). *Macromolecules*  
359 1994;27:878-80.  
360

361 Steinbüchel A and Hein S. Biochemical and molecular basis of microbial synthesis of  
362 polyhydroxyalkanoates in microorganisms. *Adv Biochem Eng Biotechnol* 2001;71:81-  
363 123.

364

365 Sudesh K, Abe H, and Doi Y. Synthesis, structure and properties of  
366 polyhydroxyalkanoates: biological polymers. *Prog Polym Sci* 2000;25:1503-1555.

367

368 Taguchi S, Yamada M, Matsumoto K et al. A microbial factory for lactate-based  
369 polyesters using a lactate-polymerizing enzyme. *Proc Natl Acad Sci USA*  
370 2008;105:17323-7.

371

372 Tappel RC, Pan W, Bergey NS et al. Engineering *Escherichia coli* for improved  
373 production of short-chain-length-*co*-medium-chain-length poly[(*R*)-3-hydroxyalkanoate]  
374 (SCL-*co*-MCL PHA) copolymers from renewable nonfatty acid feedstocks. *ACS Sustain*  
375 *Chem Eng* 2014;2;1879-1887.

376

377 Wang Q, Tappel RC, Zhu C et al. Development of a new strategy for production of  
378 medium-chain-length polyhydroxyalkanoates by recombinant *Escherichia coli* via  
379 inexpensive non-fatty acid feedstocks. *Appl Environ Microbiol* 2012;78:519-27.

380

381 Wong YM, Brigham CJ, Rha C et al. Biosynthesis and characterization of  
382 polyhydroxyalkanoate containing high 3-hydroxyhexanoate monomer fraction from  
383 crude palm kernel oil by recombinant *Cupriavidus necator*. *Bioresour Technol*  
384 2012;121:320-7.

385

386 Zhang J, Shishatskaya EI, Volova TG et al. Polyhydroxyalkanoates (PHA) for therapeutic

387 applications. *Mater Sci Eng C Mater Biol Appl* 2018;86:144-50.

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391 **Tables**

392

393 Table 1. Synthesis of the GL-based polymers containing 3HHx.

No.	Precursor Conc.* <sup>1</sup> (g L <sup>-1</sup> )			CDW (g L <sup>-1</sup> )	Polymer production (g L <sup>-1</sup> )	Monomer composition (mol%)			Local GL fraction (mol%)	Molecular weight		
	GL	3HB	3HHx			GL	3HB	3HHx		$M_n$ ( $\times 10^4$ )	$M_w$ ( $\times 10^4$ )	$M_w/M_n$
<b>1</b>			0.00	3.75 $\pm$ 0.11	0.37 $\pm$ 0.03	27	73	0	68	2.6	5.4	2.1
<b>2</b>			0.50	3.28 $\pm$ 0.23	0.37 $\pm$ 0.07	17	68	15	56	6.9	9.9	1.4
<b>3</b>	2.50	2.50	1.00	3.15 $\pm$ 0.20	0.45 $\pm$ 0.07	9	64	27	30	6.5	15	2.3
<b>4</b>			1.50	2.45 $\pm$ 1.22	0.37 $\pm$ 0.14	5	59	36	-* <sup>2</sup>	13	21	1.6

394 \*<sup>1</sup>Concentrations of precursors are shown as sodium salts. \*<sup>2</sup>The local GL fraction of **4**395 could not be calculated due to the low signal intensity. CDW: cell dry weight,  $M_w$ :396 weight average molecular weight,  $M_n$ : number average molecular weight. -: Not tested397 due to poor cell growth. Values are the average  $\pm$  standard deviation of data from three

398 independent experiments.

399

400 Table 2. Mechanical properties of GL-based PHAs.

No.	Film composition	Tensile strength (MPa)	Young's modulus (MPa)	Elongation to break (%)
-*	P(20 mol% GL- <i>co</i> -3HB)	16	348	7
2	P(17 mol% GL- <i>co</i> -3HB- <i>co</i> -15 mol% 3HHx)	1.3	63	8
3	P(9 mol% GL- <i>co</i> -3HB- <i>co</i> -27 mol% 3HHx)	1.0	7.2	43

401 \*This polymer was synthesized and reported in our previous study (Arai 2020).

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403

404 Table 3. Thermal properties of the synthesized polymers.

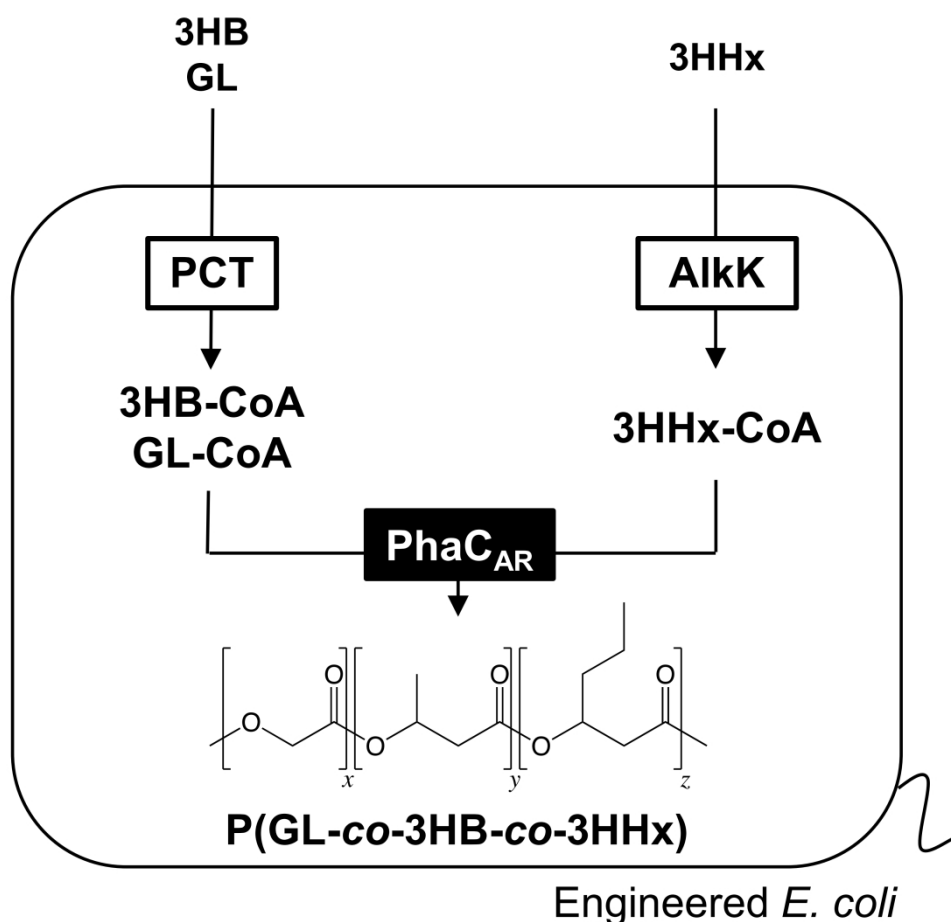
Polymer	Monomer composition (mol%)			$T_g$ (°C)	$T_m$ (°C)	$\Delta H$ (J g <sup>-1</sup> )
	GL	3HB	3HHx			
<b>1</b>	27	73	0	-0.6	157.6	43.5
<b>2</b>	17	68	15	-2.5	118.9	21.3
<b>3</b>	9	64	27	-5.2	116.9	2.3
<b>4</b>	5	59	36	-10.5	ND	ND

405

ND, Not detected.

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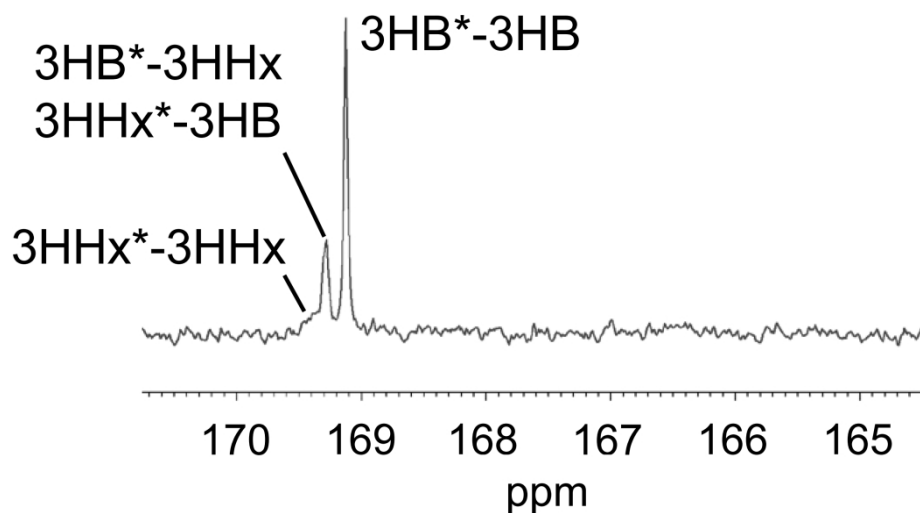


408

409 Figure 1. Illustration of the metabolic pathway used in this study to synthesize the  
 410 terpolymer P(GL-co-3HB-co-3HHx) in *E. coli*. Propionyl-CoA transferase (PCT)  
 411 transfers CoA from acetyl-CoA to GL and 3HB, thereby producing GL-CoA and 3HB-  
 412 CoA. Acyl-CoA ligase (AlkK) is an ATP-dependent enzyme that activates 3HHx into  
 413 3HHx-CoA. PhaC<sub>AR</sub> polymerizes the CoA thioesters.

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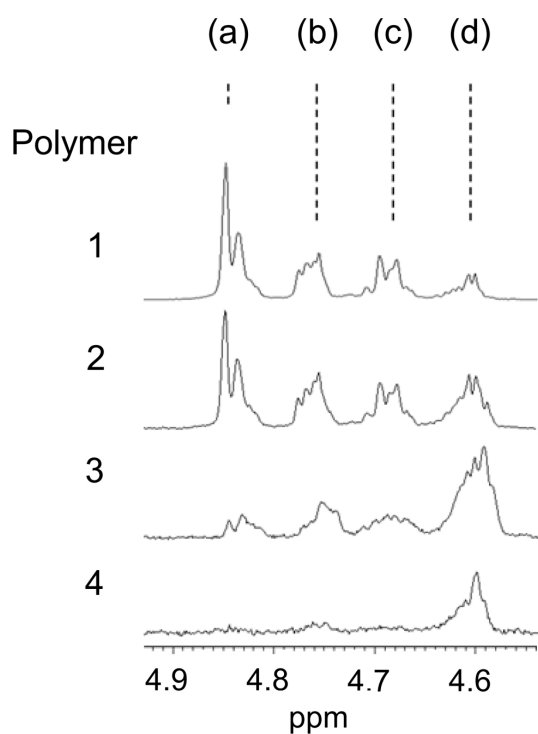


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417 Figure 2.  $^{13}\text{C}$  NMR spectrum of polymer 2. Peaks correspond to the indicated dyads. GL-

418 containing dyad peaks appear at  $\delta$  165-167. The overall spectrum is shown in Fig. S1.

419



420

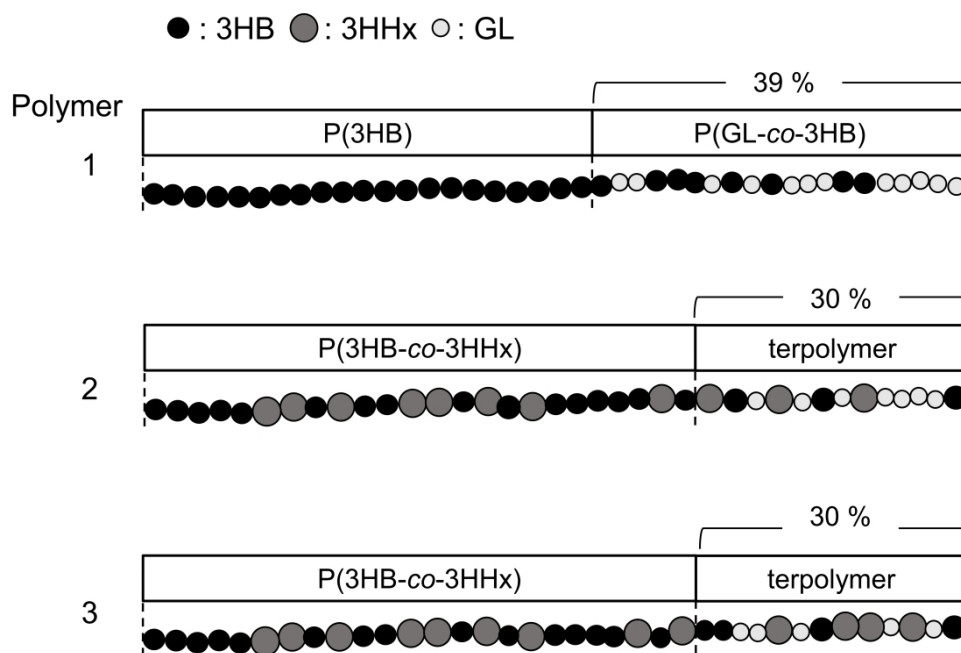
421 Figure 3. Partial  $^1\text{H}$  NMR spectra of polymers 1-4. Peaks (a)-(d) correspond to the

422 monomer triads: GL-GL\*-GL (a), GL-GL\*-(3HB/3HHx) and (3HB/3HHx)-GL\*-GL

423 ((b) or (c)), and (3HB/3HHx)-GL\*-(3HB/3HHx) (d), respectively. The full spectra are

424 shown in Fig. S3.

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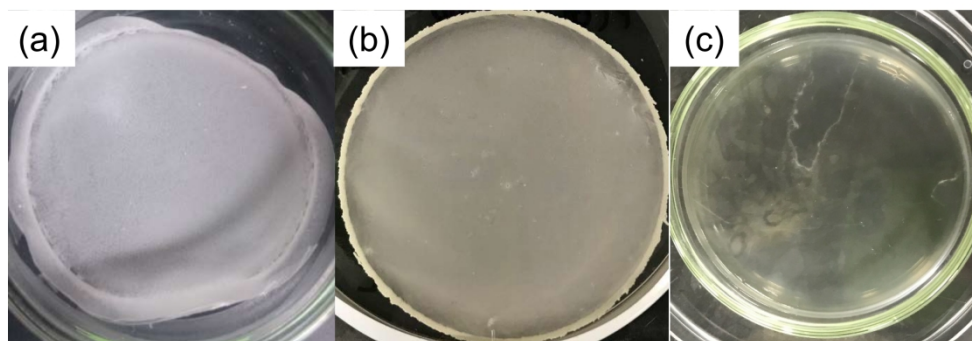


426

427 Figure 4. Proposed sequence structures of P(GL-co-3HB-co-3HHx) synthesized using

428 PhaCAR. The number and the order of the segments are not determined.

429



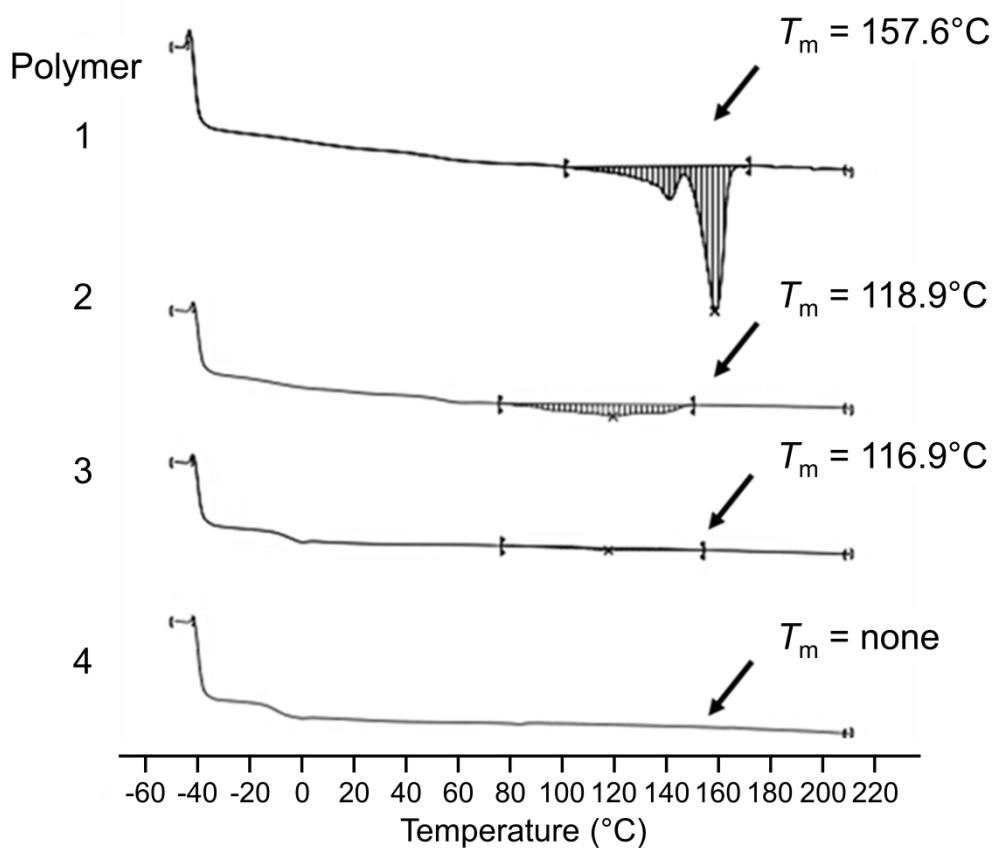
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431 Figure 5. The appearance of the solvent-cast films. (a) P(20 mol% GL-co-3HB) (Arai

432 2020), (b) P(17 mol% GL-co-3HB-co-15 mol% 3HHx) (**2**), and (c) P(9 mol% GL-co-

433 3HB-co-27 mol% 3HHx) (**3**).

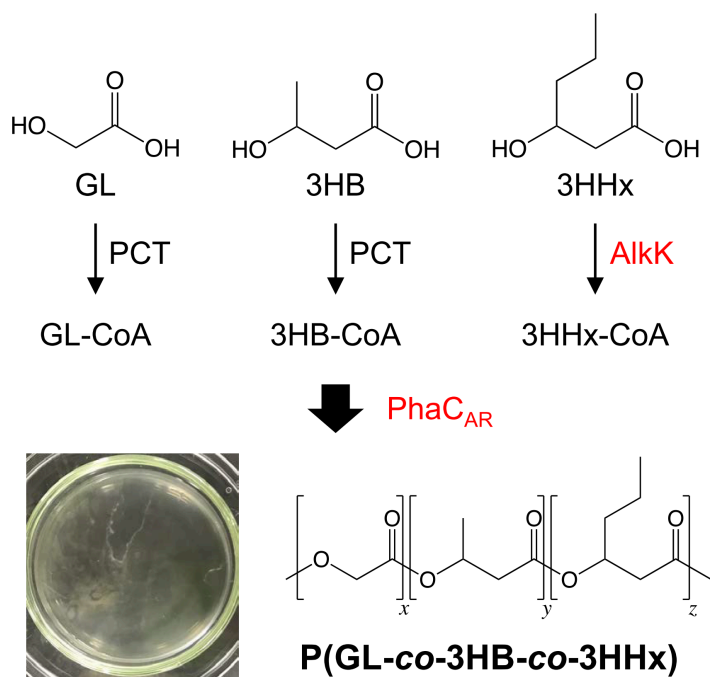
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436 Figure. 6. DSC analysis of the synthesized polymers.

437



438

439 Graphical Abstract Caption

440 A novel terpolymer P(GL-co-3HB-co-3HHx) was produced using PCT and AlkK for  
 441 monomer supply and PhaCAR for polymerization in *Escherichia coli*.

442 **Supplementary materials**

443

444 Table S1. The effects of *alkK* on the incorporation of 3HHx into the polymer.

Plasmid	Precursor Conc.* (g L <sup>-1</sup> )			CDW (g L <sup>-1</sup> )	Polymer production (g L <sup>-1</sup> )	Monomer composition (mol%)		
	GL	3HB	3HHx			GL	3HB	3HHx
	pBSPRephaC <sub>AR</sub> pct	2.5	2.5			0.0	3.19 ± 0.02	0.26 ± 0.01
1.0				2.79 ± 0.12	0.36 ± 0.00	19	81	0
pBSPRephaC <sub>AR</sub> pctAlkK	2.5	2.5	0.0	3.49 ± 0.05	0.42 ± 0.02	23	77	0
			1.0	3.30 ± 0.19	0.47 ± 0.01	9	65	26

445 \*Concentrations of precursors are shown as sodium salts. CDW: cell dry weight. Values

446 are the average ± standard deviation of data from three independent experiments.

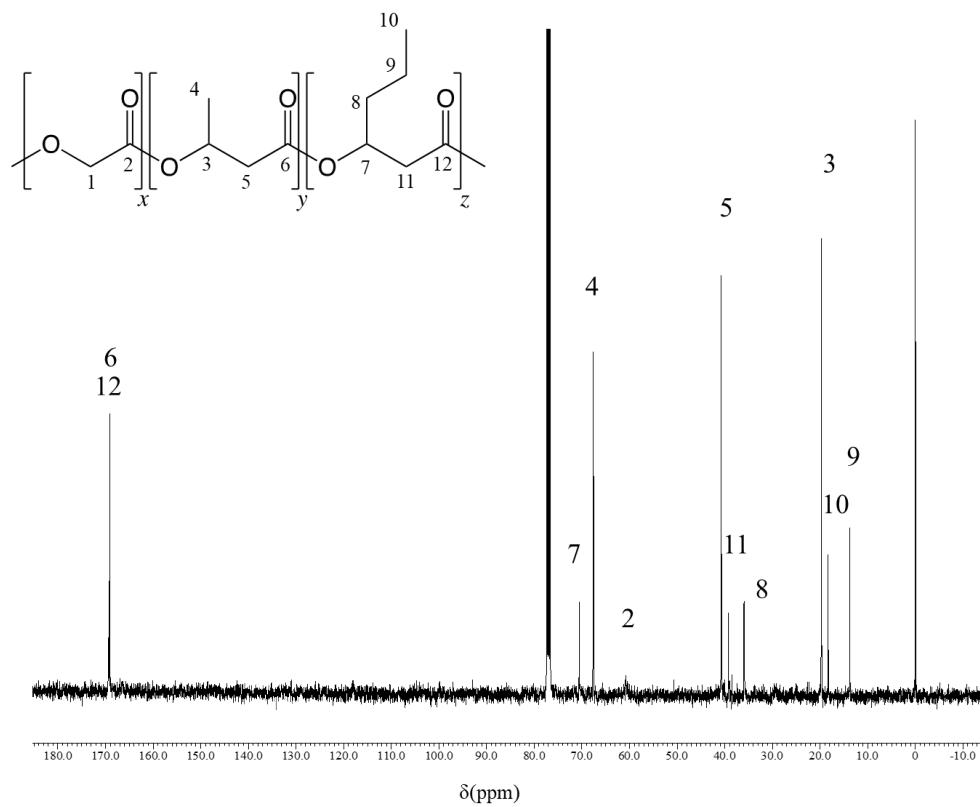
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448

449 Table S2. Monomer sequence analysis based on <sup>1</sup>H NMR.

	GL ratio (mol%)	3HB and 3HHx ratio (mol%)	Relative intensity of GL units in the triad sequences (%) <sup>*1</sup>				Local GL fraction <sup>*2</sup>
			<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	
			Synthesized polymer <b>1</b>	27	73	46.6 ± 3.0	
Calculated ideal random polymer	68	32	46	22	22	10	
Synthesized polymer <b>2</b>	17	83	33.9 ± 9.0	21.7 ± 0.7	22.2 ± 2.8	22.3 ± 6.4	56
Calculated ideal random polymer	56	44	31	25	25	19	
Synthesized polymer <b>3</b>	9	91	13.2 ± 1.6	21.4 ± 0.9	16.0 ± 1.9	49.3 ± 1.6	30
Calculated ideal random polymer	30	70	9	21	21	49	

450 \*1 For the synthesized polymers, the values **a-d** were calculated as previously described  
451 (Arai 2020). Briefly, using the function *Area*, which is the <sup>1</sup>H NMR peak area of the  
452 molecular species, **a** is defined as Area(a)/[Area(a) + Area(b) + Area(c) + Area(d)]; **b**, **c**,  
453 and **d** are defined in the same manner. For the ideal polymers, **a** was calculated as  $x^2$ ,  
454 where  $x$  is the GL ratio (mol%); **b** and **c**, and **d** were calculated as  $x(1-x)$  and  $(1-x)^2$ ,  
455 respectively. \*2 The local GL fraction ( $x$  mol%) for the calculated ideal random polymers  
456 was calculated as the residual sum of squares of the measured values of **a-d** for the  
457 synthesized polymers and the calculated values of **a-d** for the ideal polymers. Using the  
458 function  $\Delta(y)$ , which is defined as  $[y(\text{measured}) - y(\text{calculated})]$  (where  $y = a, b, c, d$ ), the  
459 GL ratio  $x$  is defined as the value that gives the minimum of  $\Sigma(y = a, b, c, d) [\Delta(y)]^2$ . The  
460 ratio of the P(3HB-*co*-3HHx) segment [P(3HB-*co*-3HHx)] was calculated using the  
461 equation:  $[P(3HB-*co*-3HHx)] = [\text{total 3HB} + 3HHx] - [\text{total GL}] \times (1-x)/x$ .  
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Figure S1.  $^{13}\text{C}$  NMR of polymer 2.

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Figure S2a. <sup>1</sup>H NMR of polymer 1.

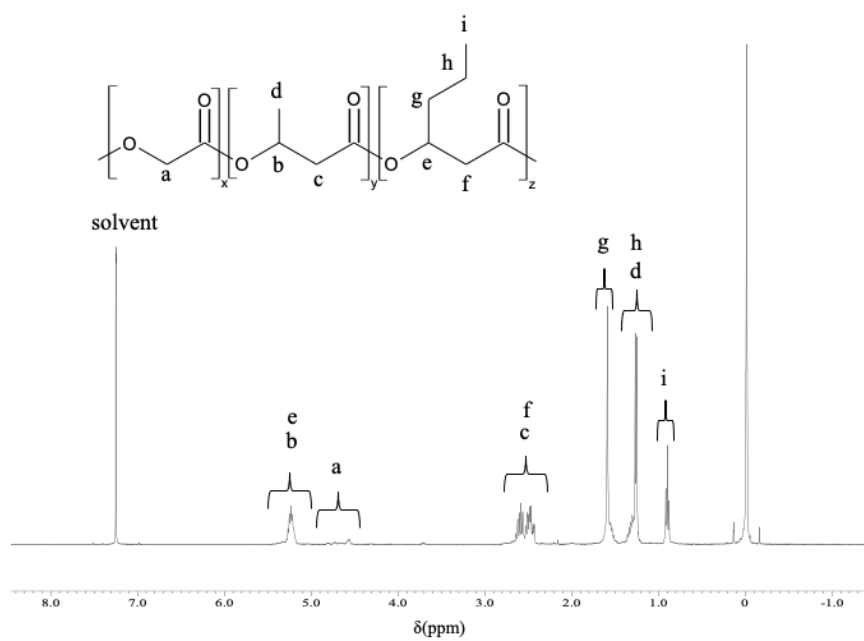


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Figure S2b. <sup>1</sup>H NMR of polymer 2.

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Figure S2c.  $^1\text{H}$  NMR of polymer 3.

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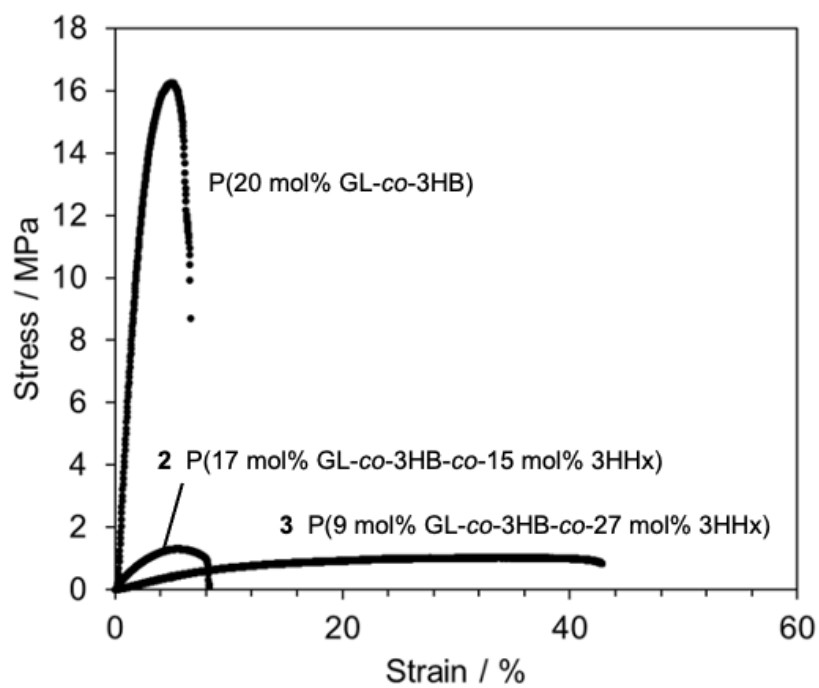
Figure S2d.  $^1\text{H}$  NMR of polymer 4.

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Figure S3. Stress-strain curve.

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