



Title	Biosynthesis of poly(glycolate-co-3-hydroxybutyrate-co-3-hydroxyhexanoate) in <i>Escherichia coli</i> expressing sequence-regulating polyhydroxyalkanoate synthase and medium-chain-length 3-hydroxyalkanoic acid coenzyme A ligase
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Citation	Bioscience, Biotechnology, and Biochemistry, 86(2), 217-223 https://doi.org/10.1093/bbb/zbab198
Issue Date	2022-02
Doc URL	http://hdl.handle.net/2115/83966
Rights	This is a pre-copied, author-produced version of an article accepted for publication in Bioscience biotechnology and biochemistry following peer review. The version of record Volume 86, Issue 2, February 2022, Pages 217–223, is available online at: https://doi.org/10.1093/bbb/zbab198 .
Type	article (author version)
File Information	Tomita_BBB_HUSCAP-1.pdf



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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_{CAR} 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_{CAR} 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_{CAR} 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_{CAR} 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_{AR}, 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_{AR} possesses unusual substrate specificity toward
54 glycolyl (GL)-CoA (Arai *et al.* 2020). We previously reported that PhaC_{AR} 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_{1Ps}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_{AR} contains 23 mol% GL, which
68 is higher than that of PhaC_{1Ps}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_{AR}.
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_{CAR} 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_{RephaCAR}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_{RephaCAR}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⁻¹ NaCl, 5 g L⁻¹
111 yeast extract, and 10 g L⁻¹ tryptone) containing ampicillin (100 mg L⁻¹) 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⁻¹, 2.5 or 5.0 g L⁻¹, and 0-2.5 g L⁻¹, 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 ¹H NMR and ¹³C NMR analyses of the extracted polymer in CDCl₃ 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⁻¹, 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⁻¹,
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_{AR}, 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 ¹³C 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 δ 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 ^1H 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 ¹³C NMR results suggest the
222 presence of a P(3HB-*co*-3HHx) segment while ¹H 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_{AR} 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_{1Ps}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

273 **Acknowledgments and Funding**

274 This work was supported by the JST-Mirai Program (No. JPMJMI19EB) and
275 JSPS Kakenhi (20H04368).

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**

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388

389

390

391 **Tables**

392

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

No.	Precursor Conc.* ¹ (g L ⁻¹)			CDW (g L ⁻¹)	Polymer production (g L ⁻¹)	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
1			0.00	3.75 \pm 0.11	0.37 \pm 0.03	27	73	0	68	2.6	5.4	2.1
2			0.50	3.28 \pm 0.23	0.37 \pm 0.07	17	68	15	56	6.9	9.9	1.4
3	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
4			1.50	2.45 \pm 1.22	0.37 \pm 0.14	5	59	36	-* ²	13	21	1.6

394 *¹Concentrations of precursors are shown as sodium salts. *²The 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).

402

403

404 Table 3. Thermal properties of the synthesized polymers.

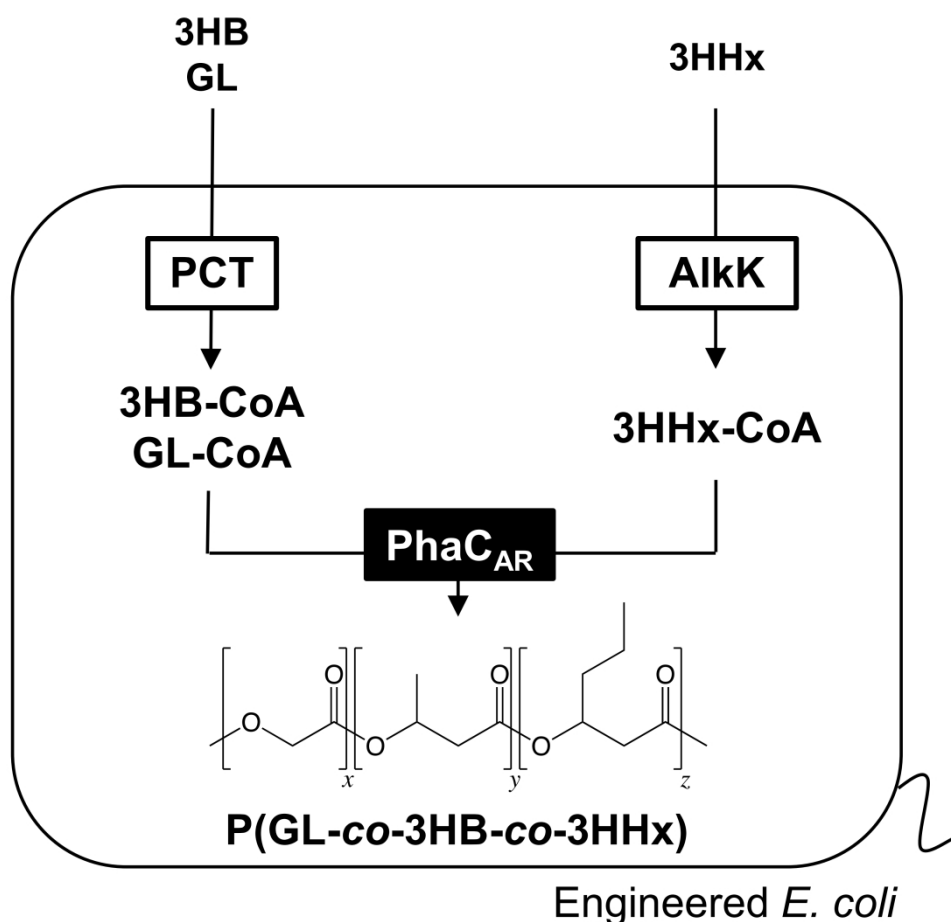
Polymer	Monomer composition (mol%)			T_g (°C)	T_m (°C)	ΔH (J g ⁻¹)
	GL	3HB	3HHx			
1	27	73	0	-0.6	157.6	43.5
2	17	68	15	-2.5	118.9	21.3
3	9	64	27	-5.2	116.9	2.3
4	5	59	36	-10.5	ND	ND

405

ND, Not detected.

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407



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)

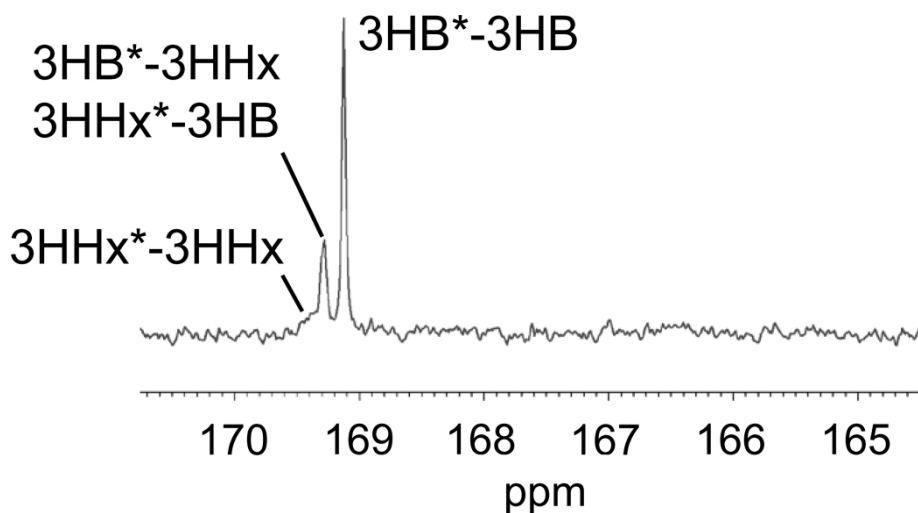
411 transfers CoA from acetyl-CoA to GL and 3HB, thereby producing GL-CoA and 3HB-

412 CoA. Acyl-CoA ligase (AlkK) is an ATP-dependent enzyme that activates 3HHx into

413 3HHx-CoA. PhaC_{AR} polymerizes the CoA thioesters.

414

415

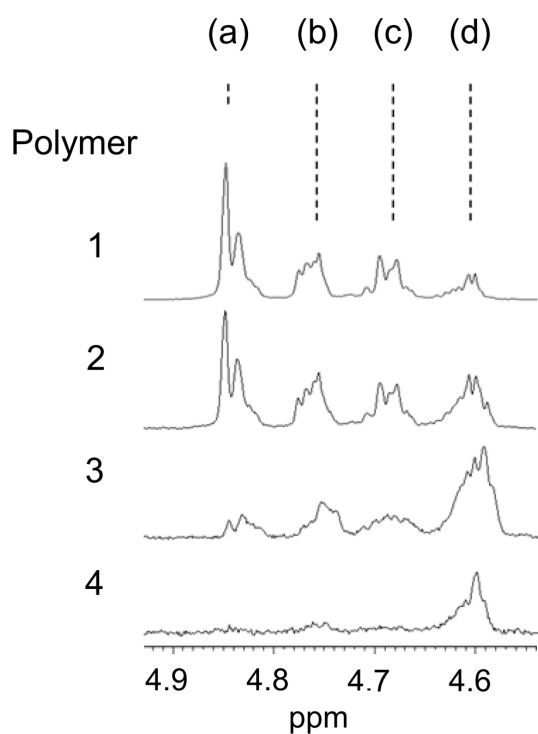


416

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

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

419



420

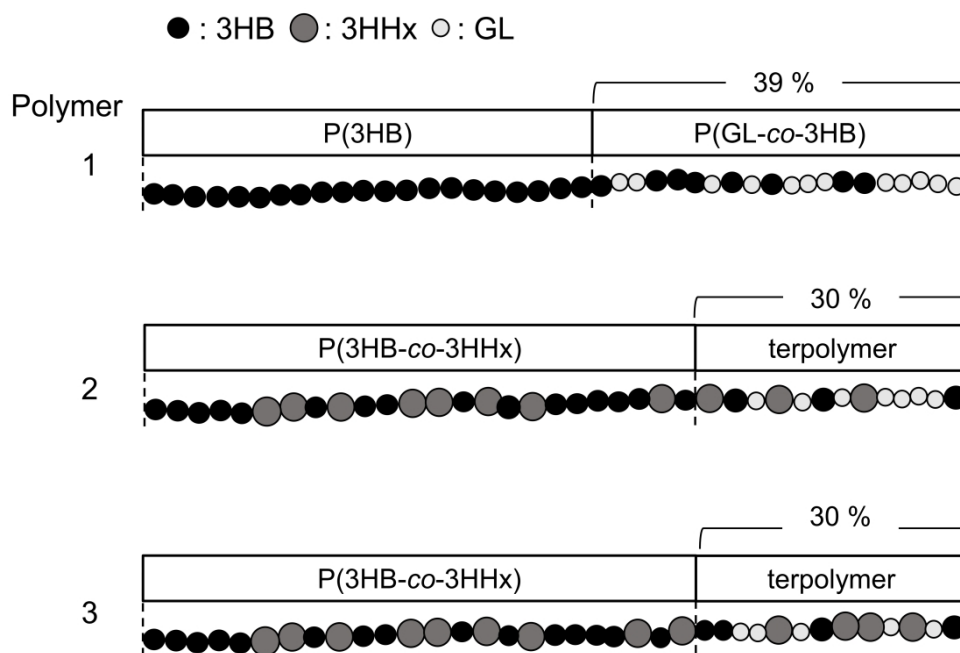
421 Figure 3. Partial ^1H NMR spectra of polymers 1-4. Peaks (a)-(d) correspond to the

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.

425

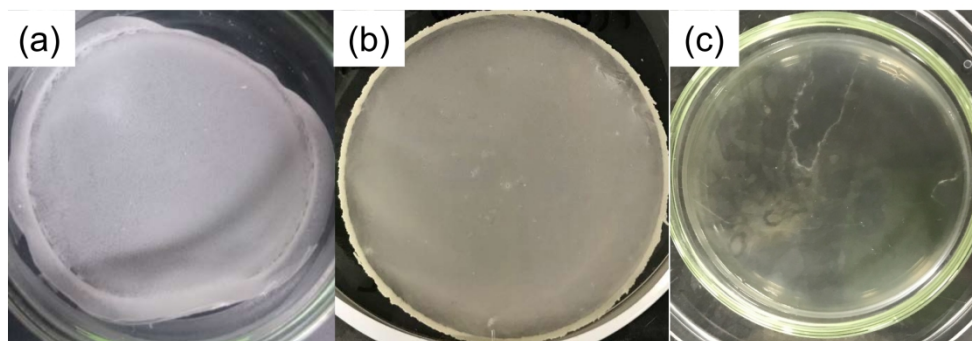


426

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



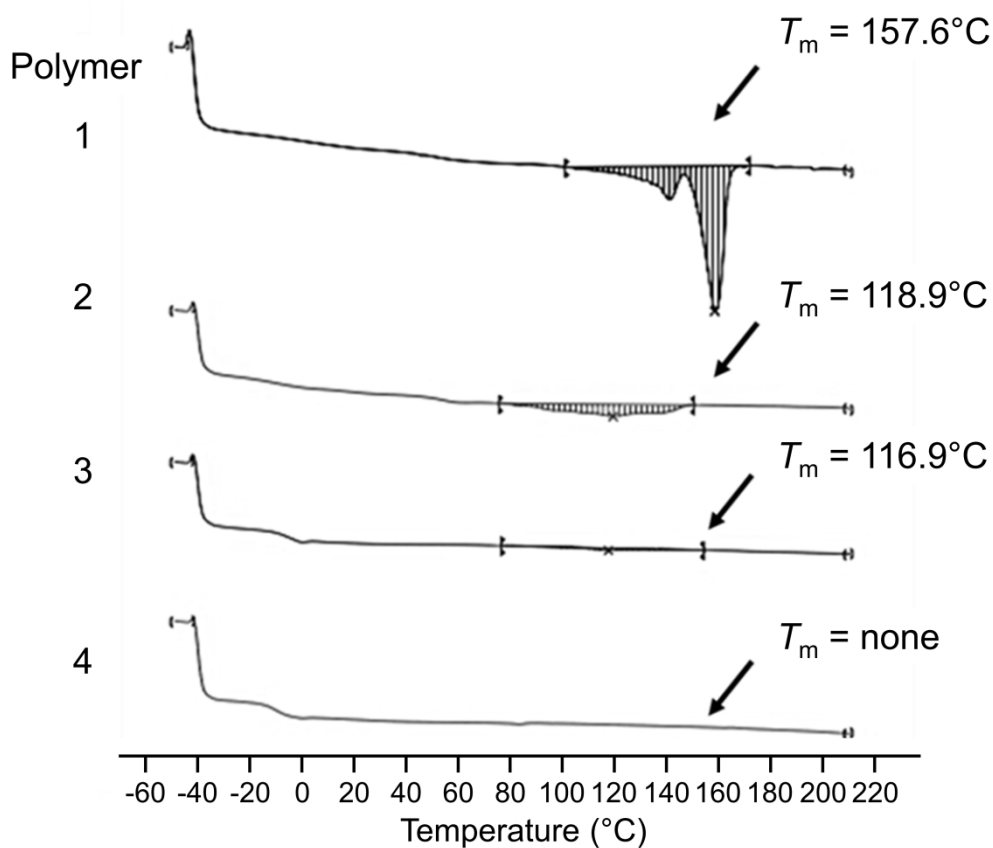
430

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).

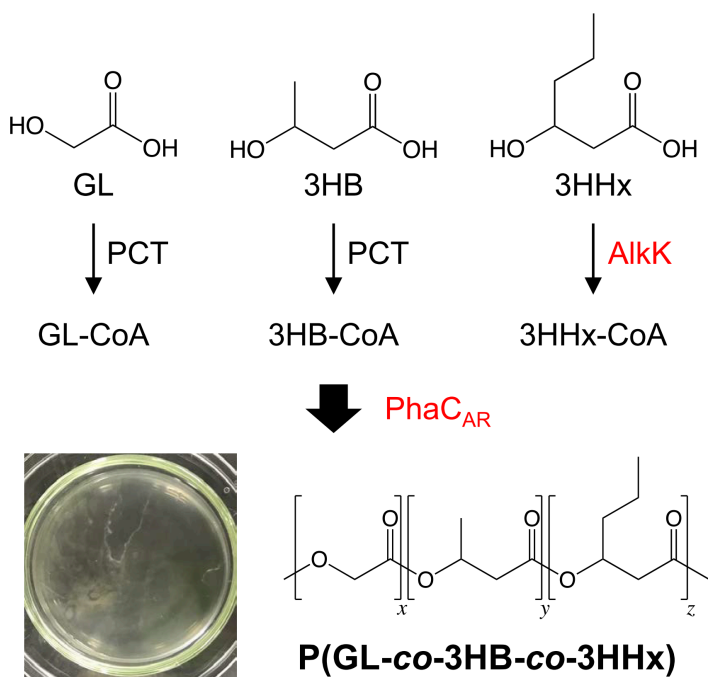
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435

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 ⁻¹)			CDW (g L ⁻¹)	Polymer production (g L ⁻¹)	Monomer composition (mol%)		
	GL	3HB	3HHx			GL	3HB	3HHx
	pBSPRephaC _{AR} 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 _{AR} 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.

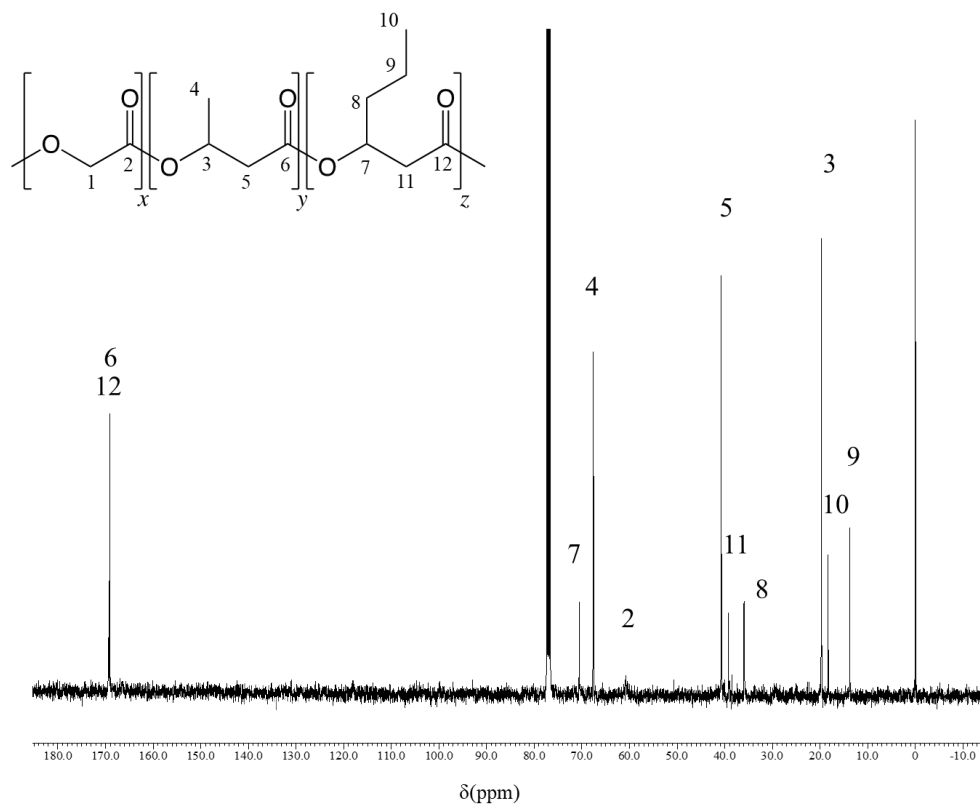
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448

449 Table S2. Monomer sequence analysis based on ¹H NMR.

	GL ratio (mol%)	3HB and 3HHx ratio (mol%)	Relative intensity of GL units in the triad sequences (%) ^{*1}				Local GL fraction ^{*2}
			<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	
Synthesized polymer 1	27	73	46.6 ± 3.0	21.0 ± 0.2	22.7 ± 2.0	9.8 ± 1.3	68
Calculated ideal random polymer	68	32	46	22	22	10	
Synthesized polymer 2	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 3	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 ¹H 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: $[P(3HB-*co*-3HHx)] = [\text{total 3HB} + 3HHx] - [\text{total GL}] \times (1-x)/x$.
462

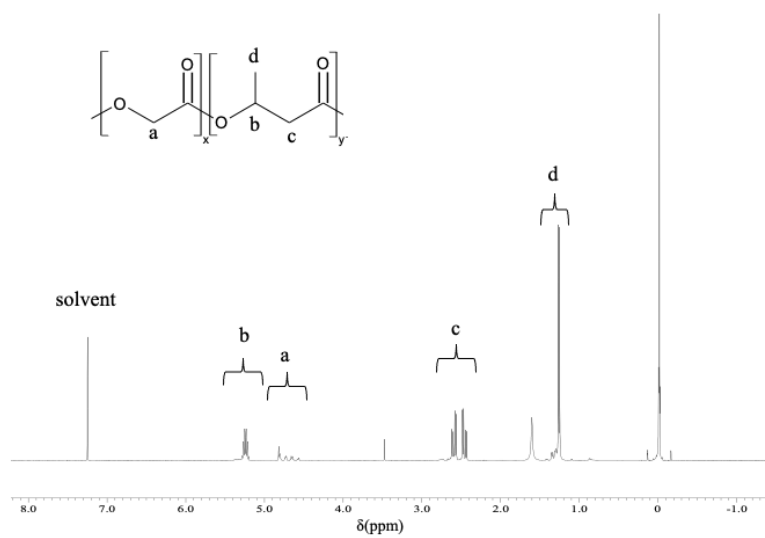


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464

Figure S1. ^{13}C NMR of polymer 2.

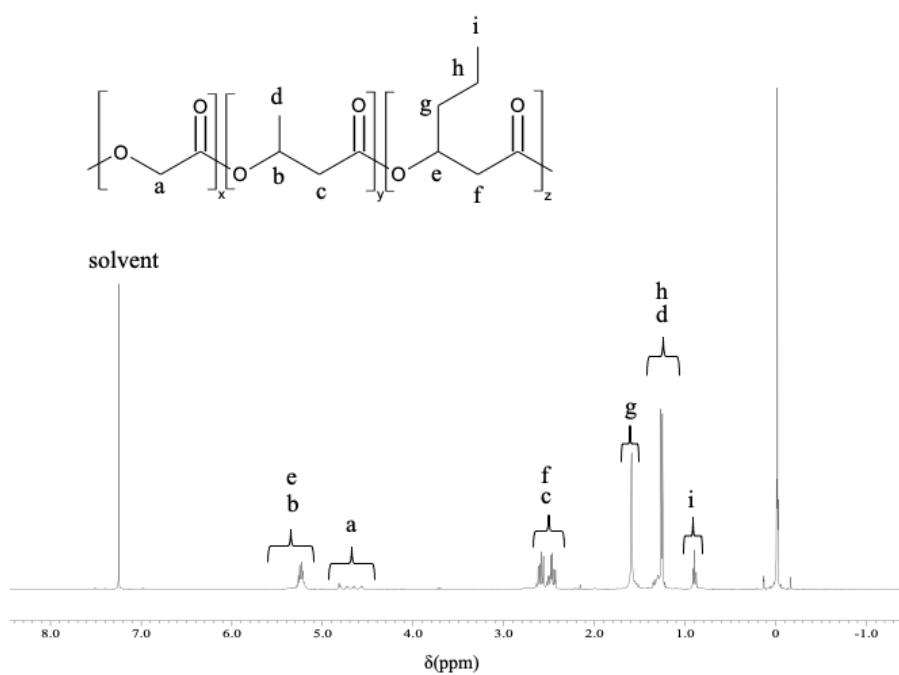
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Figure S2a. ^1H NMR of polymer 1.

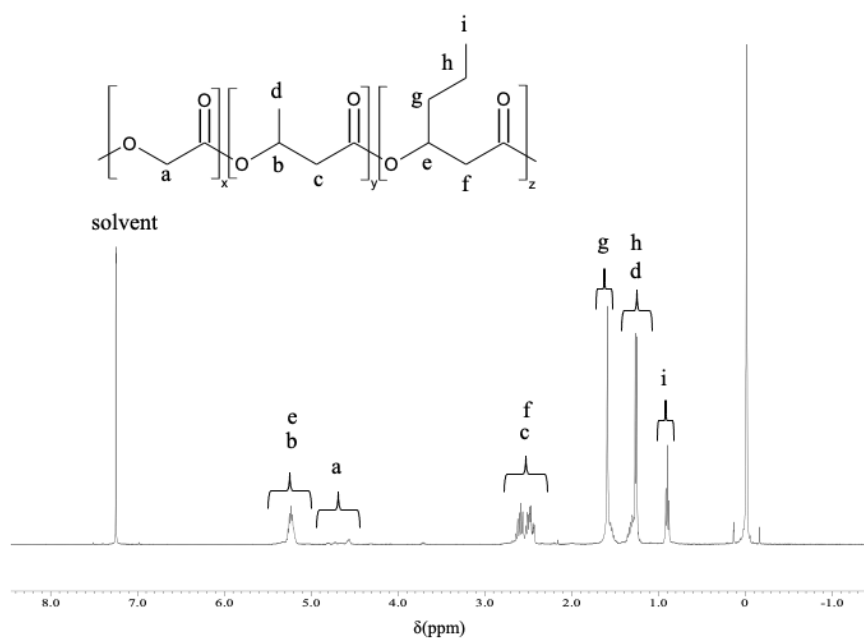


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Figure S2b. ^1H NMR of polymer 2.

470

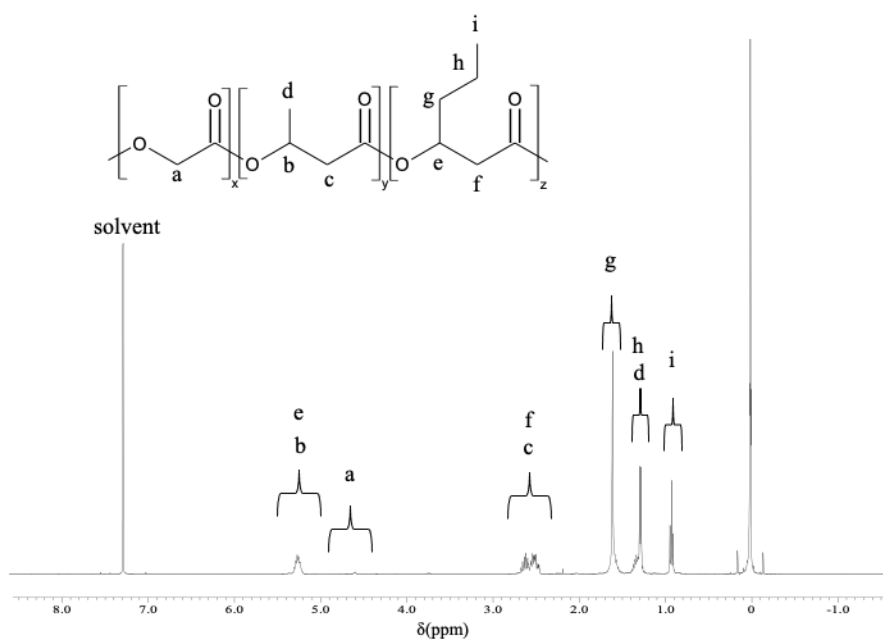


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Figure S2c. ¹H NMR of polymer 3.

473



474

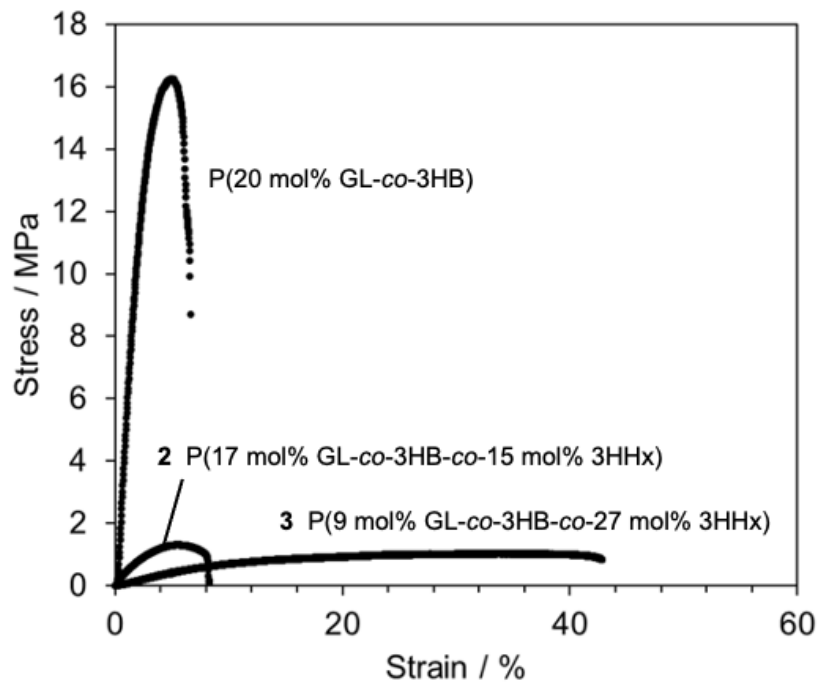
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Figure S2d. ¹H NMR of polymer 4.

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Figure S3. Stress-strain curve.

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