Real-time NMR analysis of polyhydroxyalkanoate synthase reaction that synthesizes block copolymer comprising glycolate and 3-hydroxybutyrate

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Highlights

- Real-time NMR was applied to the PHA synthase assay for the first time.
- P(3HB)-*b*-P[glycolate (GL)-*ran*-3HB] copolymer was synthesized *in vitro* by $PhaC_{AR}$.
- P(3HB) segment was synthesized prior to the P(GL-*ran*-3HB) segment.
- $PhaC_{AR}$ slightly synthesized P(GL) homopolymer.

Abstract

The sequence-regulating polyhydroxyalkanoate (PHA) synthase $PhaC_{AR}$ spontaneously synthesizes the homo-random block copolymer, poly[3-hydroxybutyrate (3HB)]-*b*-poly[glycolate (GL)-*ran*-3HB]. In this study, a real-time *in vitro* chasing system was established using a high-resolution 800 MHz nuclear magnetic resonance (NMR) and ^{13}C-labeled monomers to monitor the polymerization of GL-CoA and 3HB-CoA into this atypical copolymer. Consequently, $PhaC_{AR}$ initially consumed only 3HB-CoA and subsequently consumed both substrates. The structure of the nascent polymer was analyzed by extracting it with deuterated hexafluoro-isopropanol. In the primary reaction product, a 3HB-3HB dyad was detected, and GL-3HB linkages were subsequently formed. According to these results, the P(3HB) homopolymer segment is synthesized prior to the random copolymer segment. This is the first report of its kind which proposes the application of real-time NMR to a PHA synthase assay, paving the way for elucidating the mechanisms of PHA block copolymerization.

Keywords:

Polyhydroxybutyrate; Enzymatic polymerization; Block copolymer; Glycolate-based polymer; Polyglycolic acid

1 Introduction

Polyhydroxyalkanoate (PHA) synthases (PhaCs) are polymerases for microbial PHA production. Typically, the enzymes recognize (*R*)-3-hydroxyacyl-CoAs (3HA-CoAs) as substrates and polymerize them into homopolymers and/or copolymers [1–4]. Class I PhaCs possess substrate scope toward short-chain-length (SCL, $\leq C_5$) 3HA-CoAs, whereas class II PhaCs prefer medium-chain-length (MCL, $C_{6–12}$) substrates. Thus, PhaC is a crucial enzyme in determining polymer structure [5].

$PhaC_{AR}$ is an engineered class I PhaC with the ability to spontaneously synthesize block copolymers from a mixture of multiple substrates [6]. Additionally, $PhaC_{AR}$ can incorporate unusual 2-hydroxyalkanoate (2HA) units, such as glycolate (GL) and 2-

hydroxybutyrate (2HB), in addition to long-main-chain (C_{4-6}) hydroxyalkanoates (HAs) [7,8]. Block copolymer segments are homopolymers, random copolymers, or a combination of both. The combination of 2HA-CoA and 3HA-CoA is required for the block copolymerization by $PhaC_{AR}$. For example, $PhaC_{AR}$ synthesized poly(3-hydroxybutyrate)-*b*-poly(2HB) [P(3HB)-*b*-P(2HB)] comprising two homopolymer segments and P(3HB)-*b*-P(GL-*ran*-3HB) containing homopolymer and random copolymer segments using recombinant *Escherichia coli* grown on the corresponding monomer precursors [7]. Recently, the directed evolution of $PhaC_{AR}$ has successfully increased its activity toward the MCL monomer 3-hydroxyhexanyl-CoA [9].

The block copolymerization by $PhaC_{AR}$ is thought to proceed with an unusual mechanism because other PHA synthases synthesize random copolymers [10,11]. To date, the kinetics of the PHA synthase has been analyzed only in homopolymer synthesis conditions [12,13]. In order to perform a block copolymerization assay at the molecular level, we previously developed an LC-MS-based *in vitro* assay method, which facilitated the monitoring of the consumption of multiple substrates and the formation of free CoA in the reaction mixture [6]. An important conclusion obtained from the measurement was the order of the segments in the block copolymers, i.e., HO-[A]-[B]-COOH or HO-[B]-[A]-COOH. In the copolymerization of 3HB-CoA and 2HB-CoA by $PhaC_{AR}$, 3HB-CoA was consumed exclusively in the initial stage of the reaction, followed by 2HB-CoA consumption, indicating that the obtained polymer is HO-P(3HB)-*b*-P(2HB)-COOH [6] (Fig. 1). The mechanism responsible for switching segment synthesis is not fully understood.

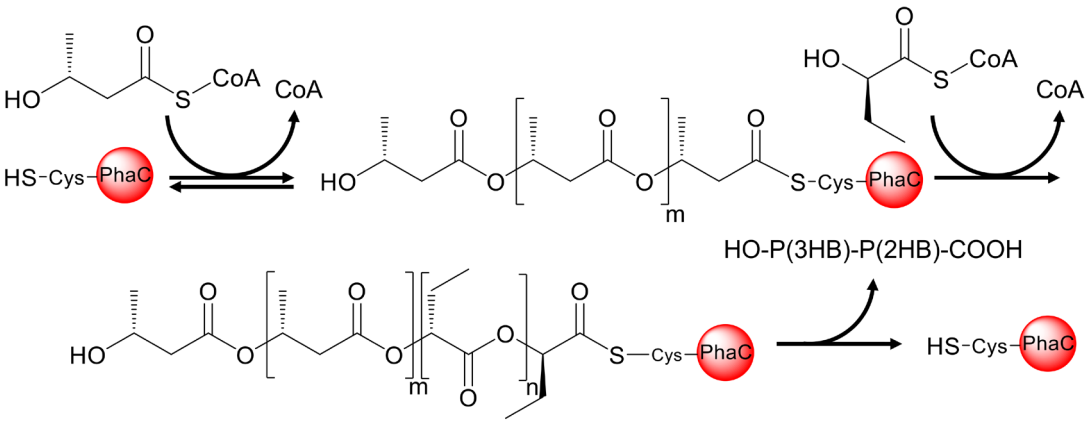

Fig. 1 P(3HB-*block*-2HB) block copolymerization model of $PhaC_{AR}$.

This study aimed at determining the segment order of P(3HB-*b*-P(GL-*ran*-3HB).

Here the simultaneous consumption of 3HB-CoA and GL-CoA is not sufficient evidence of random segment synthesis in this instance. Therefore, the monomer sequence analysis of the *in vitro* polymerization products is necessary to confirm the synthesis of a random copolymer segment. Due to the fact that the resonances of the polymerization products were weaker than those of the PhaC protein, ^{1}H NMR is hardly applicable for this purpose. Therefore, we prepared ^{13}C-labeled 3HB-CoA and GL-CoA and measured ^{1}H-^{13}C Heteronuclear Single Quantum Correlation (HSQC) spectroscopy of the reaction product, which significantly increased the signal intensities of the reaction products and permitted analysis of their fine structure, including their monomer sequences.

Moreover, we discovered that the use of labeled substrates and a high-resolution 800 MHz NMR made it possible to perform the reaction in an NMR machine and monitor the substrate consumption in real-time by measuring continuous ^{1}H-^{13}C Heteronuclear Multiple Quantum Correlation (HMQC). The NMR measurement in real-time is applicable to the conditions of copolymerization. This paper presents the first NMR analysis of *in vitro* PHA synthesis using PhaC$_{AR}$ in real-time. An engineered class II PhaC1(STQK) from *Pseudomonas* sp. 61-3 with a pairwise point mutation (S325T/Q481K) was evaluated for comparison. This enzyme is the first discovered 2HA-CoA polymerizing enzyme and synthesizes P(GL-*co*-3HB) random copolymers [14].

2 Materials and methods

2.1 Plasmids

pBSP$_{Re}$phaC$_{AR}$pct and pBSP$_{Re}$pct are derivatives of pBluescript KS^{+} containing the ampicillin resistance gene [6]. pBSP$_{Re}$phaC$_{AR}$pct contains the *phaC$_{AR}$* and *pct* genes under the P$_{Re}$ promoter from the *phb* operon in *R. eutropha*. pBSP$_{Re}$phaC1(STQK)pct contains the *phaC1*(STQK) gene from *Pseudomonas* sp. 61–3 with S325T/Q481K mutations. This plasmid was constructed as follows. The *phaC1*(STQK) was amplified using the primers 5'-CGAATAGTGACTCGAGTCTAGAAATAATTTTGTTTAACTTT-3′ and 5'-TACCGTCGACCTCGACGTCAGTAATTGTGTAGTCCTTTC-3′. Next, using the In-Fusion reaction, the amplified fragment was inserted into pBSP$_{Re}$pct that had been digested with XhoI (Takara-Bio, Japan).

2.2 Determination of intracellular monomer concentrations

Recombinant *E. coli* JM109 containing pBSP$_{Re}$pct, pBSP$_{Re}$phaC$_{AR}$pct, or pBluescript KS^{+} (empty vector) was grown at 30 °C for 18 h in two 1.5 mL LB media containing 5 g/L GL-Na (JUNSEI, Japan, chemical purity: 99.0%), 5 g/L (*R*)-3HB-Na (KANTO KAGAKU, Japan, chemical purity: 99.6%), 2 wt% glucose, and 100 mg/L

ampicillin. As previously described, the cells in a test tube was subjected to intermediate analysis [15]. The supernatant is discarded after the cells have been harvested by centrifugation (4 °C, 13,000 *g*, 5 min). The cell pellet was resuspended in 100 μL of ice-cold water and subsequently combined with 500 μL of ice-chilled acetonitrile containing 0.1 M formic acid. The suspension was disrupted by ultrasonication (UD-211, TOMY SEIKO Co. LTD., Japan) using a cup horn unit for 7 min at 1 s intervals in an iced water bath, followed by centrifugation at 13,000 *g* for 10 min at 4 °C. At 4 °C, the supernatant was condensed to 100 μL in a vacuum. LC-MS was applied to the filtered sample. LC-MS analysis was performed using an LCMS-2020 (Shimadzu, Japan) equipped with a Mastro C18 column (150 mm, Shimadzu), electrospray ionization (ESI), and single quadrupole mass spectroscopy. Carrier A: 5 mM ammonium acetate (pH 5.6) containing 5 mM dimethylbutylamine [16] and carrier B: methanol were used in gradient mode with a flow rate of 0.2 mL/min, as follows: 0 min, 10% B; 3 min, 10% B; 16 min, 94.4% B; 19 min, 94.4% B; 19.01 min, 10% B; 24 min, 10% B. The ESI voltage was 4.5 kV in the negative mode. Nitrogen was used as a nebulizer (1.5 mL/min) and drying gas (15.0 mL/min). Acetyl-CoA, GL-CoA, and 3HB-CoA were detected and quantified using the $[M-H]^-$ ions with the following *m/z* values and retention times: acetyl-CoA (*m/z* = 808, retention time: 11.8 min), GL-CoA (*m/z* = 824.2, rt.: 11.0 min), and 3HB-CoA (*m/z* = 852.2, rt.: 11.8 min).

2.3 Chemical preparation of CoA thioesters

As previously described, (*R*)-3-hydroxybutyryl-CoA (3HB-CoA) was synthesized (chemical purity: 98.9%) from (*R*)-3-hydroxybutyric acid (KANTO KAGAKU, Japan, chemical purity: 99.6%) to prepare CoA thioesters [9]. Briefly, 1,1′-carbonyldiimidazole (21.1 mg) was dissolved in 1 mL of dry tetrahydrofuran (THF) under an atmosphere of nitrogen. (*R*)-3-Hydroxybutyric acid (20 mg) was then added, and the mixture was incubated for 30 min (with stirring) at room temperature. CoA (40 mg) (Oriental Yeast, Tokyo, Japan) was dissolved in 0.5 mL of 0.5 M $NaHCO_3$ (pH 7.4), combined with the aforementioned THF solution and incubated on ice for 12 h while stirring. The reaction mixture was diluted with 4.5 mL of 0.5 M $NaHCO_3$. After acidification with formic acid to a pH of approximately 3, THF was evaporated *in vacuo*. The product 3HB-CoA was purified using preparative high-performance liquid chromatography equipped with a reverse-phase column (ODS-80Ts, TOSOH, Japan) using 0.1% formic acid and acetonitrile as the mobile phases. The fractions containing 3HB-CoA were collected, and acetonitrile was evaporated *in vacuo*. The purity of 3HB-CoA was confirmed by LC-ESI-MS as described above. The concentration of 3HB-CoA was determined by UV at 259

nm using a molar attenuation coefficient ε = 1.64 × 10^4 M^{-1} cm^{-1}, which is ε for acetyl-CoA. Glycolyl-CoA (GL-CoA) (chemical purity: 91.2%), [1,2-^{13}C]3HB-CoA (chemical purity: 89.5%), and [2-^{13}C]GL-CoA (chemical purity: 83.1%) were synthesized in the same manner using 40 mg of GL (JUNSEI, Japan, chemical purity: 99.0%), 11.4 mg of sodium [1,2-^{13}C](*R*)-3-hydroxybutyrate (ISOTEC, USA, 99 atom% ^{13}C, chemical purity: 97%), and 7.9 mg of [2-^{13}C]GL (ISOTEC, 99 atom% ^{13}C, chemical purity: 97%), respectively. All substrates purity were analyzed by LC-MS (Fig. S1).

2.4 Expression and purification of recombinant PhaC$_{AR}$ and PhaC1(STQK)

E. coli JM109(DE3) strain was utilized. Previously, the plasmids pQE30-phaC1(STQK) and pQE30-phaC$_{AR}$ encoding the N-terminally His$_6$-tagged PhaC1(STQK) and PhaC$_{AR}$, respectively, were constructed previously. pGro7 containing the *gro*ES-*gro*EL (TAKARA, JAPAN) reportedly increased the expression level of PHA synthases [17]. Cells were cultivated in 2 mL LB medium with 100 μg/mL ampicillin and 30 μg/mL chloramphenicol at 37 °C for 14 h. In order to induce GroEL-ES expression, the seed culture was transferred to 100 mL Terrific Broth (Difco Laboratories) containing 100 μg/mL ampicillin, 30 μg/mL chloramphenicol, and L-arabinose (0.5 mg/mL). At 37 °C, the cells were grown until their optical density at 600 nm (OD_{600}) was approximately 0.5 for PhaC$_{AR}$ and 0.25 for PhaC1(STQK). After the medium had cooled to 20 °C, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to induce PhaCs expression.

For PhaC$_{AR}$, cells were further cultured at 20 °C for 16 h, harvested by centrifugation (4 °C, 5,000 *g*, 10 min), resuspended in 20 mL lysis buffer (20 mM HEPES-NaOH (pH 8.0), 200 mM NaCl, and 10% glycerol), and disrupted by ultrasonication (UD-211, TOMY SEIKO Co. LTD., Japan) equipped with a tip probe in ice-chilled water for 20 min at 1-s intervals. After centrifugation (4 °C, 20,000 *g*, 20 min), soluble cell extracts were added to His60 Ni Superflow resin (Takara, Japan) (500 μL suspension), mixed gently at 4 °C for 30 min, and then applied to an open column. In a stepwise gradient, His-tagged PhaC$_{AR}$ was eluted with lysis buffer containing 50–500 mM imidazole. Subsequently, using a PD-10 column (GE Healthcare, USA), the buffer was replaced with 50 mM sodium phosphate (pH 7.4) containing 0.05% methyl-6-*O*-(*N*-heptylcarbamoyl)-α-D-glucopyranoside (Hecameg, GE Healthcare) (store buffer). The concentration of protein was determined using the Bradford method. All procedures were conducted at 4 °C.

PhaC1(STQK) was initially purified using the same procedure as PhaC$_{AR}$. The protein was then subjected to size exclusion chromatography utilizing a HiLoad 26/60

Superdex 200 pg column (GE Healthcare) with 50 mM sodium phosphate (pH 7.0) containing 150 mM NaCl as the mobile phase at a flow rate of 2 mL/min. The column was calibrated using a gel filtration standard (Biorad, Hercules, CA, USA). As described previously, the fractions containing PhaC1(STQK) were collected and subjected to nickel affinity chromatography once more to concentrate the protein. The protein was eluted using the previously mentioned lysis buffer containing 500 mM imidazole, and the buffer was exchanged to 50 mM sodium phosphate (pH 7.4) containing 0.05% Hecameg and 5% glycerol using a PD-10 column.

2.5 *In vitro* enzymatic reaction for LC-MS analysis

A microtube containing a reaction mixture containing 5 mM ammonium acetate (pH 7.0) and different concentrations of substrate(s) was preincubated at 30 °C for 5 min. In order to initiate the reaction, purified PhaCs were added (final concentration of 0.1 mg/mL for $PhaC_{AR}$ and 0.25 mg/mL for PhaC1(STQK)), followed by incubation at 30 °C for a maximum of 6 h. The volume total was 600 µL. Additionally, 50 µL aliquots were periodically transferred to other tubes and combined with 50 µL of 1% trichloroacetic acid to stop the reaction. The tubes were centrifuged at 20,000 *g* at 4 °C for 10 min, and the supernatant was analyzed using LC-MS. One unit of enzyme activity is the amount required to catalyze the transformation of one micromole of the substrate in 1 min.

2.6 *In vitro* enzymatic reaction for NMR analysis

For real-time NMR analysis, a reaction mixture (650 µL) containing 5 mM ammonium acetate (Sigma-Aldrich, USA, chemical purity 99.0%) and 200 µM ^{13}C-labeled substrate(s) in 5% D_2O (ISOTEC, 99.9 atom% D) in an NMR 5 mm tube was preincubated at 30 °C in the NMR probe. D_2O is required for NMR frequency locking, but 5% was added to mitigate its impact on the enzyme reaction. After thermal equilibration, the reaction mixture was removed from the NMR machine, combined with 50 µL of $PhaC_{AR}$ (final concentration 50 µg/mL) to initiate the reaction, and then returned to the NMR machine for continuous measurement. 700 µL was the final volume of the reaction mixture. The zero point of the NMR spectrum of the reaction mixture was measured using HMQC mode with water suppression using a 3–9–19 type watergate sequence to eliminate the water signal. The pulse program will be published elsewhere. The NMR data were recorded every 15 min using BRUKER AVANCE NEO 800 MHz spectrometers with a cryogenic probe, CPTCI(F) (Bruker).

Following real-time NMR analysis, sample solutions were lyophilized for polymer product analysis. Samples were dissolved in 120 µL 1,1,1,3,3,3-hexafluoro-2-propanol-

d_2 (HFIP-d_2) (Wako, Japan, 98 atom% D) and incubated for 1 h at room temperature. HSQC phase-sensitive Echo/Antiecho-TPPI gradient selection was utilized in the NMR analysis [18–20].

2.7 Polymer structure analysis in the early stage of the reaction

For analyzing polymer structure in the early stage of the reaction, a reaction mixture containing 5 mM ammonium acetate and 200 μM ^{13}C-labeled substrate(s) in a microtube was preincubated at 30 °C for 5 min. Then, PhaC$_{AR}$ was added (final concentration of 50 μg/mL) to the reaction mixture to initiate the reaction. The final volume was 1 mL. After 5 min of incubation at 30 °C, 1 mL of 1% trichloroacetic acid was added to stop the reaction. The mixture was lyophilized, and the dried sample was resuspended in 120 μL HFIP-d_2 before HSQC measurements were performed.

3 Results

3.1 Intracellular concentrations of 3HB-CoA and GL-CoA in *E. coli.*

In order to determine the initial conditions of the *in vitro* experiments, i.e., the concentrations of 3HB-CoA and GL-CoA, the intracellular monomer levels of P(GL-*co*-3HB)-producing and relevant *E. coli* were measured. The intracellular monomer levels were evaluated as a relative value to that of acetyl-CoA as a reference [21].

Under conditions of 3HB supplementation alone, a 3HB-CoA pool was detected in pBSP$_{Re}$pct-containing cells, indicating the functional expression of PCT (Fig. 2a). There is no significant difference between the levels of 3HB-CoA in cells expressing PhaC1(STQK) and PCT and cells expressing PCT alone. Therefore, PhaC1(STQK) is likely a rate-determining enzyme in the flux toward P(3HB). In contrast, 3HB-CoA levels were lower in cells expressing PhaC$_{AR}$ and PCT, which may be because PhaC$_{AR}$ is more active toward 3HB-CoA than PhaC1(STQK).

Under conditions of co-supplementation with 3HB and GL, the 3HB-CoA and GL-CoA levels in cells expressing PCT alone were comparable (Fig. 2b). This indicates that the intracellular 3HB-CoA and GL-CoA levels are similar at the equilibrium point of PCT-catalyzed reactions. The 3HB-CoA level in PhaC$_{AR}$-expressing cells was lower than that of PhaC1(STQK) and 3HB-supplemented conditions alone. In contrast, there was no difference between PhaC1(STQK) and PhaC$_{AR}$ GL-CoA levels.

Based on the relative intracellular concentrations of 3HB-CoA and GL-CoA, concentrations in the same magnitude were used as initial conditions for the *in vitro* PhaC assay with 3HB-CoA and GL-CoA.

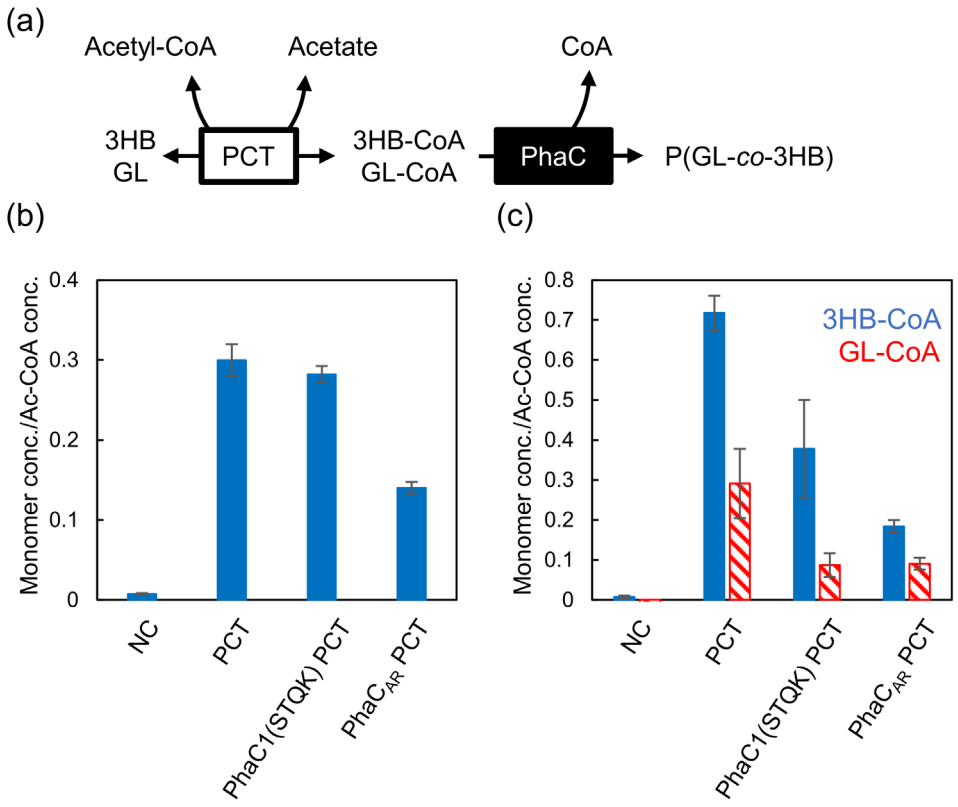

Fig. 2 The pathway for P(GL-*co*-3HB) synthesis in *E. coli* (a). Intracellular levels of 3HB-CoA and GL-CoA in *E. coli* were grown with the addition of 3HB only (b) and 3HB and GL (c). The intermediate levels are indicated relative to the concentration of acetyl-CoA. 3HB: solid blue bar, GL: hatched red bar. NC: empty plasmid pBSKS$^+$, PCT: pBSP$_{Re}$pct, PhaC1(STQK)PCT: pBSP$_{Re}$phaC1(STQK)pct, and PhaC$_{AR}$PCT: pBSP$_{Re}$phaC$_{AR}$pct. Error bars indicate standard deviations of three independent trials.

3.2 *In vitro* analysis of the consumption of 3HB-CoA and GL-CoA by PhaCs using LC-MS

The reactions of PhaC$_{AR}$ and PhaC1(STQK) with 3HB-CoA and GL-CoA were monitored *in vitro* using the LC-MS method. For PhaC$_{AR}$, when 3HB-CoA was used as the sole substrate, it was rapidly consumed within 5 min (317 U/μg protein) (Fig. 3a), and free CoA was released. In contrast, when GL-CoA was used alone, there was no consumption for 30 min, and then consumption occurred gradually (Fig. 3b). In the combined 3HB-CoA and GL-CoA condition (1:1 M ratio) (Fig. 3c), the 3HB-CoA concentration began to decrease 15 min after the 5-min lag phase at a rate of 118 U/μg protein, while GL-CoA was consumed at a relatively slower rate (10.6 U/mg protein). When GL-CoA concentration was decreased (3HB-CoA:GL-CoA = 7:1 M ratio), the rate

of 3HB-CoA consumption increased (418 U/μg protein) (Fig. 3d). In contrast, the consumption of 3HB-CoA slowed considerably with increasing GL-CoA concentration (16.9 U/μg protein) (Fig. 3e, 3HB-CoA:GL-CoA = 1:3 M ratio). The presence of 3HB-CoA accelerated the GL-CoA reaction, whereas GL-CoA had a negative effect on the 3HB-CoA reaction. The delayed consumption of GL-CoA indicates that the P(3HB) segment was initially synthesized. Small amounts of GL-CoA and 3HB-CoA co-consumption were observed. Therefore, the synthesis of the random copolymer segment was not conclusively determined.

Next, the same experiments were conducted with PhaC1(STQK). When 3HB-CoA or GL-CoA was the only substrate, these substrates were consumed, and free CoA was released (Fig. 3f, g). Initial consumption rate of GL-CoA (16.8 U/μg protein) was greater than that of 3HB-CoA (4.62 U/μg protein), but the reaction was terminated before GL-CoA was depleted. When a mixture of substrates was employed, both were consumed at comparable rates (13.0 and 7.11 U/μg protein for GL-CoA and 3HB-CoA, respectively; Fig. 3h), indicating that PhaC1(STQK) copolymerized the substrates, which is consistent with the results of *in vivo* experiments [22].

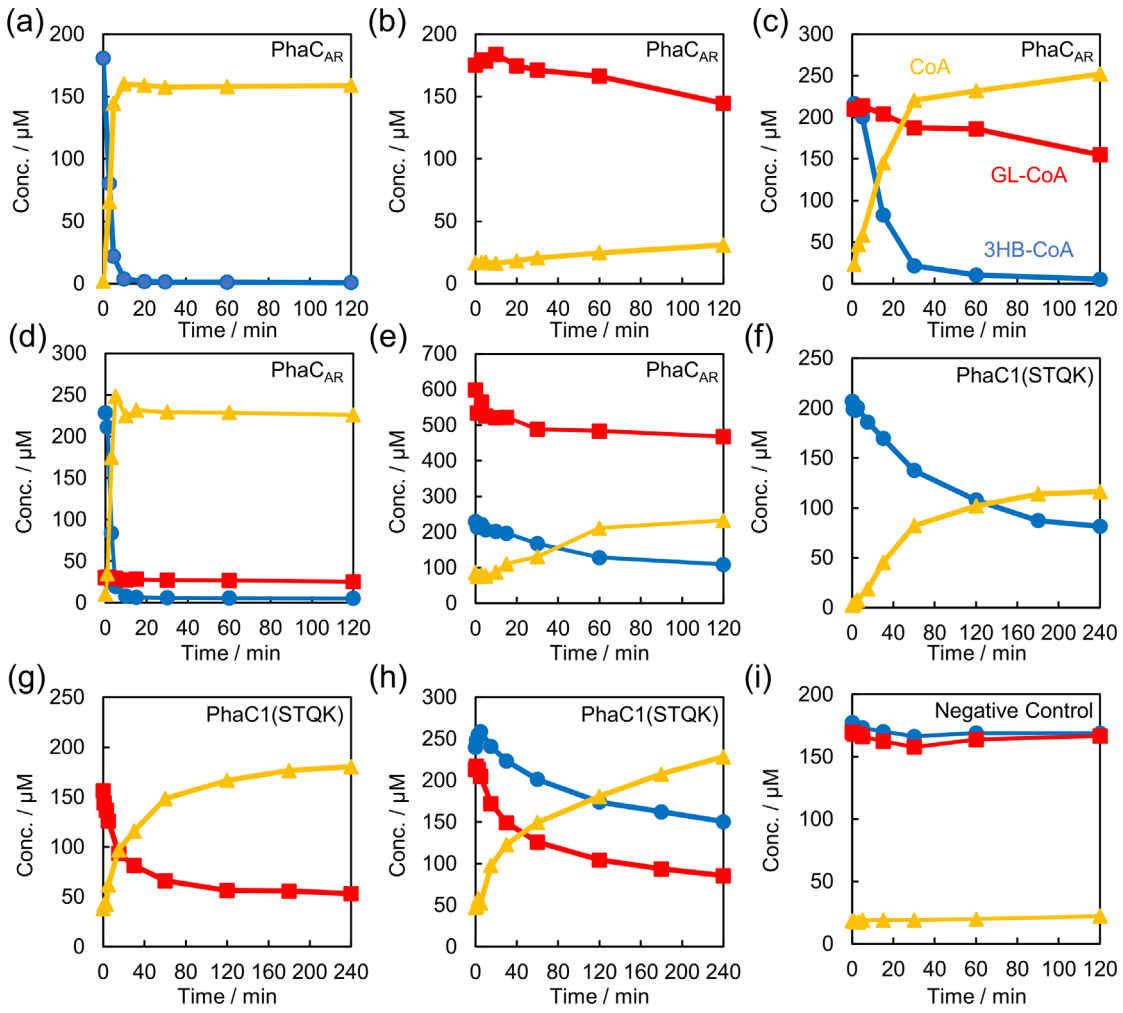

Fig. 3 LC-MS assay of PhaC$_{AR}$ and PhaC1(STQK). (a) PhaC$_{AR}$ with 3HB-CoA; (b) PhaC$_{AR}$ with GL-CoA; (c) PhaC$_{AR}$ with a 1:1 mixture of 3HB-CoA and GL-CoA; (d) PhaC$_{AR}$ with a 7:1 mixture of 3HB-CoA and 30 μM GL-CoA; (e) PhaC$_{AR}$ with a 1:3 mixture of 3HB-CoA and GL-CoA; (f) PhaC1(STQK) with 3HB-CoA; (g) PhaC1(STQK) with GL-CoA; (h) PhaC1(STQK) with a 1:1 mixture of 3HB-CoA and GL-CoA; (i) no enzyme with a 1:1 mixture of 3HB-CoA and GL-CoA (negative control). 3HB-CoA: blue cricle, GL-CoA: red square, free CoA: yellow triangle.

3.3 Monomer sequence analysis of P(GL-co-3HB) synthesized in vitro using NMR.

In order to determine the monomer sequence of *in vitro* reaction products, NMR analysis of the obtained polymer was attempted. The ^{1}H NMR resonance of the methylene proton of GL units can be used to determine the monomer sequence of a polymer [7]. However, the region's signals were not detected because they overlapped with protein signals (data not shown). Therefore, ^{13}C-labeled substrates were utilized for HSQC NMR

to increase PHA's relative resonance intensity relative to that of proteins.

Under the copolymerization condition for PhaC1(STQK), the 3HB and GL resonances were detected (Fig. 4a). The proportion of GL was 64.8 mol%. Moreover, the GL resonance splits into three or four triad signals (GL-GL*-3HB, 3HB-GL*-GL, and 3HB-GL*-3HB). The pattern of triad signals containing GL and 3HB units was previously analyzed using the copolymers synthesized *in vivo* [7]. Thus, the *in vitro* product of 3HB-CoA and GL-CoA using PhaC1(STQK) was a random copolymer P(GL-*ran*-3HB).

After 24 h under the $PhaC_{AR}$ copolymerization condition, the GL and 3HB signals were also observed (Fig. 4b). The GL resonance split into four signals that correspond to the triad sequences (GL-GL*-GL, GL-GL*-3HB, 3HB-GL*-GL, and 3HB-GL*-3HB) [7]. Based on the relative resonance intensities of split triad sequences, the local GL fraction in the P(GL-*ran*-3HB) segment was calculated (43 mol%) [7]. The total GL fraction (3 mol%) was significantly lower than the GL fraction in the region. Each segment length was estimated to be P(3HB): P(GL-ran-3HB) = 93: 7. In other words, the presence of a GL-rich region in the copolymer is indicated by the detection of the GL-GL*-GL signal. The substantial difference between the total and local GL fractions suggests the presence of the P(3HB) homopolymer segment and P(GL-*ran*-3HB) random copolymer segment in the product. These findings are consistent with our previous report on the *in vivo* synthesis of the copolymer. Furthermore, when the reaction was halted after 5 min, the P(3HB) resonance was observed, but no split GL signals were detected (Fig. 5). At 5 min, the cross signal at 4.81/59.2 ppm did not match the GL. This unequivocally demonstrates that the P(3HB) segment is synthesized before the random segment.

In the GL-CoA alone supplemented condition, the GL-GL*-GL signal was observed (Fig. S2), although the GL signal was relatively weak in comparison to the 3HB-CoA and GL-CoA co-supplemented condition. The detection of GL-GL*-GL suggests that $PhaC_{AR}$ is capable of synthesizing P(GL) [structurally identical to polyglycolic acid (PGA)]. In order to verify P(GL) synthesis *in vivo*, GL-supplemented recombinant *E. coli* cells were directly ethanolyzed and subjected to gas chromatography (GC) analysis (Fig. S3). Consequently, a GL accumulation of 2.1 wt% was detected. These findings demonstrated that $PhaC_{AR}$ synthesized the small quantity of P(GL). It is impossible to determine the molecular weight of the polymer because it is insoluble in chloroform.

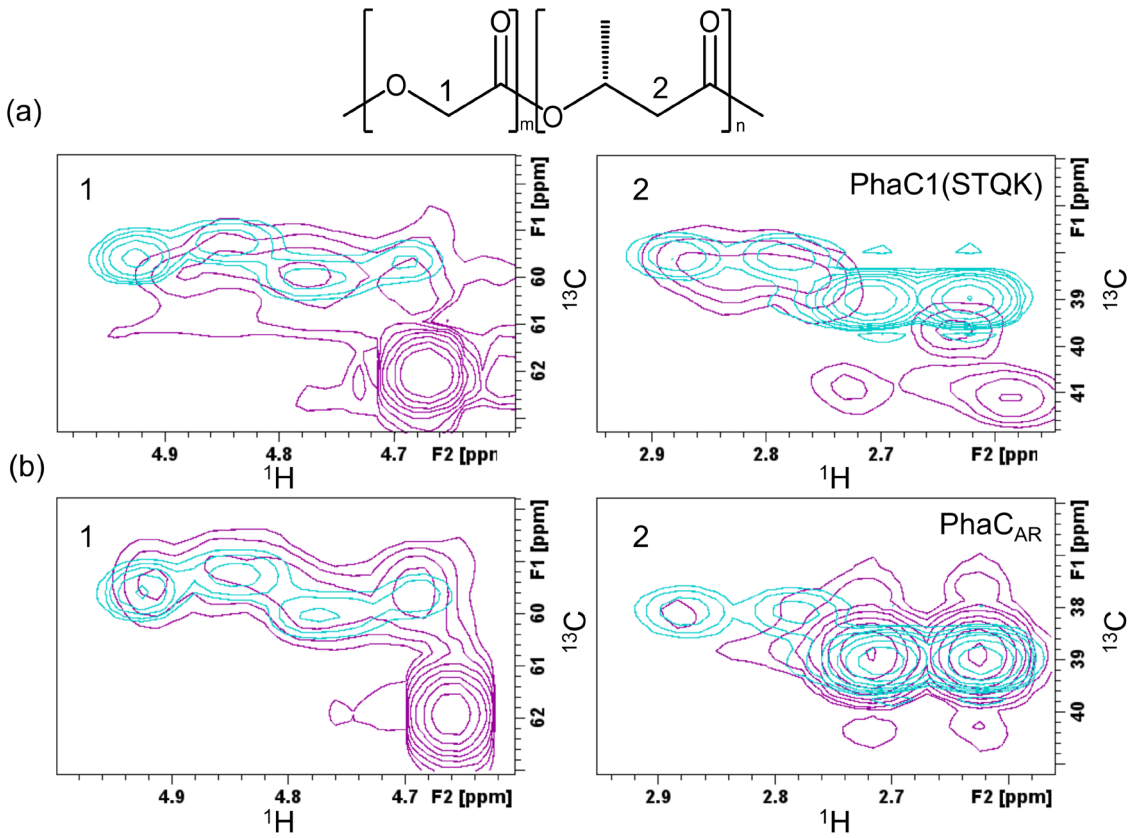

Fig. 4 HSQC NMR of *in vitro* products synthesized using PhaC1(STQK) (a) and PhaC$_{AR}$ (b) from 3HB-CoA and GL-CoA in HFIP-d_2. No.1: GL (CH_2) and No. 2: 3HB (CH_2). Purple: *in vitro* reaction products [Number of scans: 128; Acquisition time: F2 = 0.0409600 s, F1 = 0.0038552 s; recycle delay: 1.5 s; number of t1 points: 256, amount of sample used: 20.2 μg (when substrates were fully consumed), samples were collected at 24 h.]; light blue: P(3HB)-*b*-(GL-*ran*-3HB) synthesized *in vivo* (Number of scans: 4; Acquisition time: F2 = 0.0409600 s, F1 = 0.0038552 s; recycle delay: 2.0 s; number of t1 points: 256, amount of sample used: 5 mg.). HSQC 1D slices and 1D ^{1}H and 1D ^{13}C NMR spectra were shown in Fig. S4 and S5 respectively.

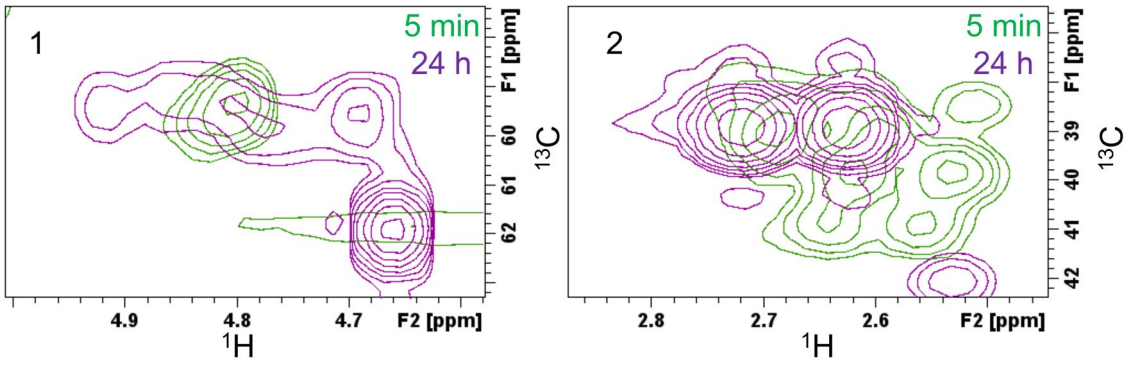

Fig. 5 HSQC NMR of the *in vitro* PhaC$_{AR}$ reaction products with 3HB-CoA and GL-CoA. No.1: GL (CH_2) and No. 2: 3HB (CH_2). Green: product at 5 min. Purple: product at 24 h. [Size of fid: F2 = 1024, F1 = 256 (real 128+imaginary 128); Spectral width: F2 = 15.6121 ppm, F1 = 165 ppm; Transmitter frequency offset: F2 = 4.7 ppm, F1 = 75 ppm; Number of scans: 32; Approximately acquisition time: F2 = 0.041 s, F1 = 0.0039 s; recycle delay: 1.5 s; number of t1 points: 256, amount of sample used: 20.2 µg (when substrates were fully consumed); zero-filling used: no.].

3.4 Real-time NMR analysis of P(GL-*co*-3HB) production *in vitro.*

The use of ^{13}C-labeled substrates, high-resolution 800 MHz NMR, and HMQC mode shortened acquisition time to 15 min. HMQC mode with water suppression using a 3–9–19 type watergate sequence was better at removing the water signal compared to HSQC method. From this accomplishment, we conceived the notion of measuring the reaction in real-time using NMR. The substrates and PhaC were combined to initiate the reaction, and the NMR machine was used to monitor changes in resonances (Fig. S8 and S9). The analysis using non-labeled substrates was not successful because of overlap with the enzyme signals (data not shown).

As the result of measurement of PhaC1(STQK) reaction with 3HB-CoA and GL-CoA at almost the same rates (Fig. 6a). The GL-CoA signal has a larger error due to the low substrate content later in the reaction, in addition to the water and enzyme buffer-derived glycerol signals. The proportion of consumed GL-CoA and 3HB-CoA (63.0 mol%) matched the monomer composition of the polymer (64.8 mol%) (Fig. 4a). This result has been reproduced with the same experiments (Fig. S10a).

PhaC$_{AR}$ consumed GL-CoA and 3HB-CoA, while the enzyme consumed 3HB-CoA faster than GL-CoA (Fig. 6b). When GL-CoA was used alone, PhaC$_{AR}$ consumed very little GL-CoA (Fig. S11). These results correspond with the LC-MS result (Fig. 3b, c). A similar consumption pattern was observed at 20 °C (Fig. S10b).

This real-time NMR method would detect all water-soluble ^{13}C-labeled compounds, including oligomers and degraded monomers. Consequently, no signal other than GL-CoA and 3HB-CoA was detected above the sensitivity threshold. Therefore, it was determined that neither PhaC$_{AR}$ nor PhaC1(STQK) produced a soluble byproduct.

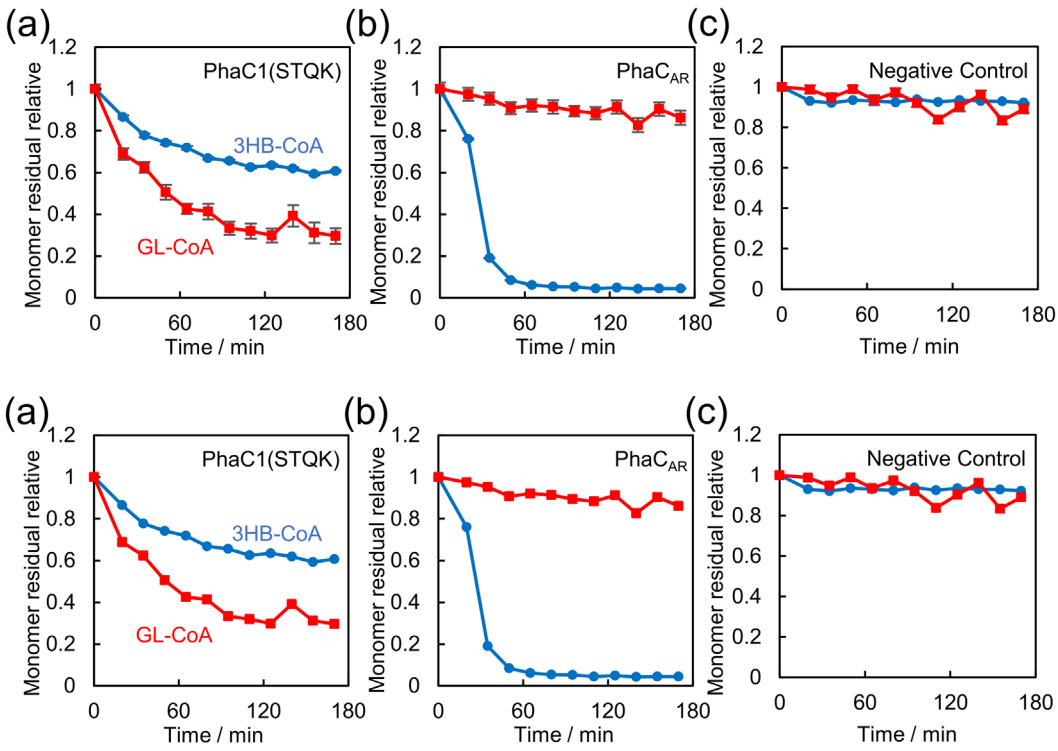

Fig. 6 Real-time NMR assay of PHA synthase reactions with ^{13}C-labeled 3HB-CoA and GL-CoA. (a) PhaC1(STQK), (b) PhaC$_{AR}$, (c) no enzyme (negative control). 3HB-CoA: blue circle, GL-CoA: red square. The y-axis values are relative methylene signal volume of ^{13}C-labeled GL-CoA and 3HB-CoA. The reaction was conducted at 30 °C. The acquisition time was 15 min using HMQC mode. The error bars indicate the noise level of each measurement calculated from the signal-to-noise ratio. [Size of fid: F2 = 2048, F1 = 64 (real 32+imaginary 32); Spectral width: F2 = 16.4432 ppm, F1 = 30 ppm; Transmitter frequency offset: F2 = 4.7 ppm, F1 =55 ppm; Number of scans: 8; Approximately acquisition time: F2 = 0.078 s, F1 = 0.0053 s; recycle delay: 1.5 s; used zero-filling].

4 Discussion

Determination of PHA copolymerization kinetics is a key methodology to understanding the mechanism of copolymer synthesis at a molecular level, particularly

with ordered monomer sequences. A conventional method for PHA synthase assay employs 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) to detect the thiol group of the released free CoA under single substrate conditions [23]. Previously, we reported the LC-MS-based PHA copolymerization assay method, which can monitor the consumption of multiple substrates [6]. The real-time NMR method described in the present study, which also determines copolymerization rates, has several advantages over the LC-MS method: the measurement is performed in the NMR machine without taking a sample from the reaction mixture, and molar ratios of multiple substrates can be determined without the complexity of quantification using ESI. In addition, it is possible to measure the water-insoluble reaction product(s), i.e., polymer(s), by extracting them with a deuterated organic solvent. However, the low temporal resolution may be a drawback. Therefore, the method is appropriate for relatively slow reactions, such as the incorporation of unusual monomer substrates. In addition, the low spectral resolution and sensitivity have been common challenges for NMR measurement. In this study, the CH_2 resonances of GL and 3HB units were observed at very different chemical shift, which facilitated the analysis.

The real-time NMR technique facilitates the detection of unidentified, water-soluble, ^{13}C-containing reaction byproducts, such as monomers and oligomers. As a result, $PhaC_{AR}$ and PhaC1(STQK) reactions under the conditions of this study produced no water-soluble byproduct. This indicates that chain-transfer reactions do not occur frequently. Miyahara et al. (2019) reported that ethanol-deficient *E. coli ΔadhE* produced water-soluble oligomeric $(3HB)_n$ with no carboxy-terminal cap, but oligomer production was not detected *in vitro* [24]. Our findings were consistent with their observation.

Our NMR analyses of *in vitro* reaction products demonstrated that $PhaC_{AR}$ produces P(3HB) prior to P(GL-*ran*-3HB). This indicates that the activity of $PhaC_{AR}$ toward GL-CoA dramatically increased during polymerization, allowing the enzyme to synthesize the random copolymer segment. Similarities exist between the phenomenon and the acceleration of the PhaC reaction. The initial slowness of the typical PhaC polymerization reactions and the progressive acceleration of polymerization have been well-established [4]. The duration of a slow reaction is known as the "lag phase." It is believed that the lag phase is caused by a requirement for priming and dimerization of PhaCs [25]. According to reports, the dimer PhaCs is composed of open and closed asymmetric conformations [13]. sT-CoA, an artificial primer, has been shown to increase $PhaC_{Re}$ activity [26]. These results suggest that $(3HB)_3$ bound to $PhaC_{Re}$ accelerates polymerization sufficiently. In contrast, the molecular weight of the P(3HB) segment in the block copolymer synthesized *in vivo* was estimated to have a molecular weight between 10^4 and 10^5 [7]. This suggests that P(3HB) with high molecular weight is required to expand the substrate specificity of

$PhaC_{AR}$. Additional research is required to elucidate the role of the P(3HB) segment in block copolymer synthesis.

The synthesis of P(GL) homopolymer by $PhaC_{AR}$ was demonstrated by *in vivo* and *in vitro* experiments. P(GL) production *in vivo* was significantly lower than that of the copolymer. Likewise, the cessation of *in vitro* reaction of GL-CoA was consistent with the observed phenomenon: PhaC1(STQK) reaction with lactyl-CoA [27]. The cessations were likely due to the low mobility of the nascent polymer chain, which could decelerate the elongation of the polymer. The glass transition temperature (T_g) is a measure of the mobility of amorphous polymer chains. P(3HB), whose T_g is 4 °C, can be efficiently synthesized at 30 °C. In contrast, P(GL) and PLA exhibit T_g values of 35–40 °C [28] and 60 °C, respectively, which are higher than the cultivation temperature, which may account for the low efficiency of P(GL) synthesis.

Author contributions

Kengo Yanagawa: Investigation, Methodology, Writing–Original draft. Ayaka Kajikawa and Sayaka Sakakibara: Investigation. Hiroyuki Kumeta: Methodology. Hiroya Tomita: Writing–Review & Editing, Funding acquisition. Ken'ichiro Matsumoto: Conceptualization, Supervision, Writing–Review & Editing, Funding acquisition.

Declaration of Competing Interest

The authors have declared that no competing interests exist.

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