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Research Article

Engineered Nanogel Particles Enhance the Photoautotrophic Biosynthesis of Polyhydroxyalkanoate in Marine Photosynthetic Bacteria

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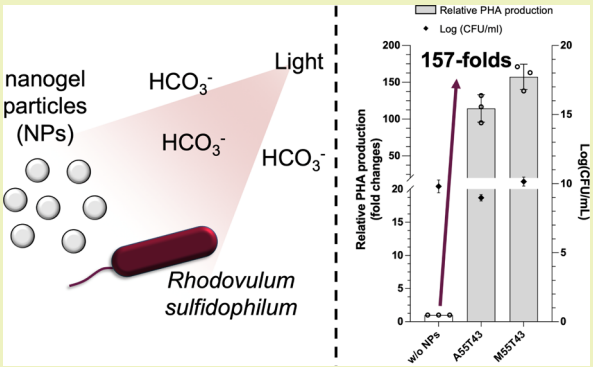
ABSTRACT: Improving polyhydroxyalkanoate (PHA, a biodegradable plastic) production under photoautotrophic cultivation is challenging for sustainable bioproduction. In this study, we demonstrated the use of engineered nanogel particles to enhance PHA accumulation in the marine photosynthetic bacterium *Rhodovulum sulfidophilum* under photoautotrophic culture. We screened the effect of 13 engineered nanogel particles on the cell growth and PHA accumulation of *R. sulfidophilum*. The addition of anionic nanogel particles significantly enhanced PHA accumulation in *R. sulfidophilum* up to 157-fold compared to that without nanogel particles. By performing ^{13}C tracer experiments and gas chromatography−mass spectrometry analysis, we confirmed that HCO_3^- was assimilated throughout the central carbon metabolism and that the accumulated PHA was indeed incorporated from HCO_3^-. Our results indicate successful PHA production with the supplementation of engineered nanogel particles under photoautotrophic cultivation in *R. sulfidophilum*. Furthermore, the strategy of using engineered nanoparticles demonstrated in this study may be applicable to other microbial cell factories to produce other commodity metabolites.



KEYWORDS: *photosynthetic bacteria Rhodovulum sulfidophilum, polyhydroxyalkanoate (PHA), photoautotrophic production, engineered nanogel particles, microbial cell factory*

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a family of biologically synthesized polymers used in a wide variety of microorganisms for energy storage. PHAs have gained much attention due to their similar thermomechanical properties to those of petrochemical-based plastics, with the additional advantages of being biodegradable and biocompatible.[1−3] PHAs have many potential applications that vary from the packaging field to biomedical materials.[3,4] PHAs are expected to replace the currently mass-produced petrochemical-based plastics to achieve sustainable development for an eco-friendly society.[3] Despite their potential for establishing a sustainable society, the major constraint in implementing PHAs for such purposes is the high-cost raw materials such as plant oil and sugars required for their production in heterotrophic organisms. Many studies have focused on producing PHAs using autotrophic organisms capable of utilizing inexpensive and abundantly available resources such as sunlight and carbon dioxide (CO_2).[5−8]

Purple nonsulfur bacteria can convert light energy and assimilate CO_2 and nitrogen, which are the three most essential traits of autotrophic organisms.[9,10] *Rhodovulum sulfidophilum* is a purple nonsulfur anoxygenic photosynthetic bacterium that naturally produces PHA.[11] It has versatile metabolic properties, including the ability to grow in high-salinity culture media (such as seawater), which lowers the risk of biological contamination, and the ability to tolerate a wide range of pH values and temperatures.[12] Its ability to grow under a high dynamic range of conditions makes it less complicated to establish a large-scale cultivation process, suggesting that *R. sulfidophilum* is a promising and cost-effective biofactory of PHA.[13] Despite the potential of *R. sulfidophilum* as a PHA biofactory, PHA is produced under heterotrophic conditions that require malate, acetate, or pyruvate as carbon sources.[14,15] A strategy to improve PHA production under photoauto-

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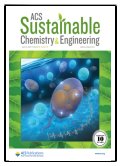

Table 1. Composition of the Engineered Nanogel Particles[a]

NPs		monomer (mol %)							
		NIPAm	DMAPM	TBAm	AAc	MAc	MMA	AMPS	BIS
nonionic	D0	98							2
	T40	58		40					2
cationic	D5	93	5						2
	D5T40	53	5	40					2
	D55T43		55	43					2
anionic	A5	93			5				2
	A5T40	53		40	5				2
	A55T43			43	55				2
	M55T43			43		55			2
	M10	88				10			2
	M20	78				20			2
	M55MMA43					55	43		2
	$SO_3$5	93						5	2

[a]Abbreviations: NPs, nanogel particles; NIPAm, *N*-isopropylacrylamide; DMAPM, *N*-[3-(dimethylamino)propyl]methacrylamide; TBAm, *N*-*tert*-butylacrylamide; AAc, acrylic acid; MAc, methacrylic acid; MMA, methyl methacrylate; AMPS, 2-acrylamido-2-methyl-1-propanesulfonic acid; and BIS, *N*,*N*′-methylenebis(acrylamide).

trophic cultivation is required to develop *R. sulfidophilum* into a sustainable PHA biofactory.

Nanogels have been widely proposed as promising carriers for drug delivery and other medical applications due to the biocompatibility and fine-tunability of their physical properties.[16] The use of engineered nanoparticles from a microbial cell factory perspective is a relatively new idea in the microbial biotechnology field. Nanoparticle engineering has been effectively applied to solve some technical issues in biorefineries, such as inducing algae flocculation for rapid harvesting,[17] enhancing light transfer,[18] inducing natural lipid and astaxanthin,[19] and carotenoid[20] production. Recently, the engineered nanogel particles that have the ability to either absorb CO_2 or capture salt ions have been developed.[21,22] These abilities are of particular interest for developing a novel cultivation method using a combinatorial strategy to combine the use of engineered nanogel particles with microbial cultivation under autotrophic conditions. However, the application of these engineered nanogel particles[21,22] has not been demonstrated in biorefinery application yet.

In this study, we aimed to investigate the effects of engineered nanogel particles on bioproduction in a microbial cell factory. We first screened the effects of 13 engineered nanogel particles on cell growth and PHA accumulation in *R. sulfidophilum*. We found that simply supplementing nanogel particles into the *R. sulfidophilum* photoautotrophic culture greatly enhanced PHA accumulation up to 157-fold. Using isotope carbon-labeling experiments, we confirmed that the PHA produced under this cultivation strategy is genuinely incorporated from $NaHCO_3$. Our results have demonstrated the application of nanogel particles to induce PHA accumulation in *R. sulfidophilum* under photoautotrophic conditions, bringing us a step closer to implementing *R. sulfidophilum* as a sustainable PHA biofactory.

METHODS

Nanogel Particle Synthesis. Thirteen engineered nanogel particles were synthesized from various monomer components (Tables 1 and S1) using the protocol described in a previous study.[21,22] Briefly, for anionic and nonionic nanogel particles, the monomers except for *N*-*tert*-butylacrylamide (TBAm) were dissolved in a certain amount of water (final monomer concentration is 312 mM). Sodium dodecyl sulfate (SDS) was also added to the solution for the anionic and nonionic nanogel particle. The solution was stirred at 70 °C under N_2 bubbling. For M55MMA43, the temperature was set to be 60 °C to avoid evaporation of methyl methacrylate (MMA). For the TBAm-containing nanogel particles, TBAm in methanol (0.64 g/mL) was injected into the heated solution afterward. Radical polymerization was initiated following the addition of 4,4′-azobis(4-cyanovaleric acid) (V-501) in dimethyl sulfoxide (10 mg/mL) or 2,2-azobis(isobutyronitrile) in methanol (42 mg/mL). Polymerization was induced at 70 °C for 3 h. For M55T43 and A55T43, polymerization was carried out for 1 h to avoid aggregation. The polymerized solution was purified by dialyzing against an excessive amount of water over 2 days. Cation-exchange resins were used to remove the counter cation. The resins were filtered out after exchanging for 30 min. The concentration was determined from the weight of NPs obtained by the lyophilization of 1−5 mL of the purified solution. The cationic nanogel particles were prepared by a similar protocol to the anionic and nonionic nanogel particles. In the polymerization, cetyltrimethylammonium bromide was used instead of SDS. Also, the radical polymerization was initiated following the addition of 2,2′-azobis(2-methylpropionamidine)dihydrochloride in water (140 mg/mL). Anion-exchange resins were used to remove the counter anion. All nanogel particles were prepared as stock solutions in purified water at a 20−25 mg/mL concentration, subjected to UV sterilization, and used for *R. sulfidophilum* cultivation.

Culture Medium Composition and Photosynthetic Bacteria Cultivation. *R. sulfidophilum* DSM1374/ATCC35886/W4, which was obtained from the American Type Culture Collection (ATCC) biological resource center (BRC), was cultured in the M6 medium containing the following composition (supplemented with 2% NaCl) per liter: sodium malate, 5 g; KH_2PO_4, 0.75 g; K_2HPO_4, 0.78 g; $CaCl_2{\cdot}2H_2O$, 0.029 g; $MgSO_4{\cdot}7H_2O$, 0.247 g; $(NH_4)_2SO_4$, 1 g; $FeSO_4{\cdot}7H_2O$, 0.011 g; vitamin solution, 10 mL; and trace element solution, 10 μL. The composition of vitamin solution per 100 mL was as follows: nicotinic acid, 0.1 g; thiamine, 0.1 g; biotin, 0.005 g; *para*-aminobenzoic acid, 0.05 g; vitamin B_{12}, 0.001 g; vitamin B_5, 0.05 g; pyridoxine HCl, 0.05 g; EDTA·3Na, 0.2 g; folic acid, 0.05 g; $ZnCl_2{\cdot}5H_2O$, 70 μg; $MnCl_2{\cdot}4H_2O$, 100 μg; H_3BO_3, 60 μg; $CoCl_2{\cdot}6H_2O$, 200 μg; $CuCl_2{\cdot}2H_2O$, 20 μg; $NiCl_2{\cdot}6H_2O$, 20 μg; and $Na_2MoO_4{\cdot}H_2O$, 40 μg. The trace element solution contained the following composition per liter: $MnSO_4{\cdot}4H_2O$, 11.16 g; $ZnSO_4{\cdot}7H_2O$, 2.88 g; $Co(NO_3)_2{\cdot}6H_2O$, 2.92 g; $CuSO_4{\cdot}5H_2O$, 2.52 g; $Na_2MoO_4{\cdot}2H_2O$, 2.42 g; H_3BO_3, 3.10 g; and EDTA·3Na, 41.20 g. The pH of the medium was adjusted to 7.0 before autoclave sterilization.

For seed culture preparation, one agar-grown colony of *R. sulfidophilum* was cultured in 50 mL of the M6 medium in sterile screw-cap tubes. The culture was maintained under continuous far-red

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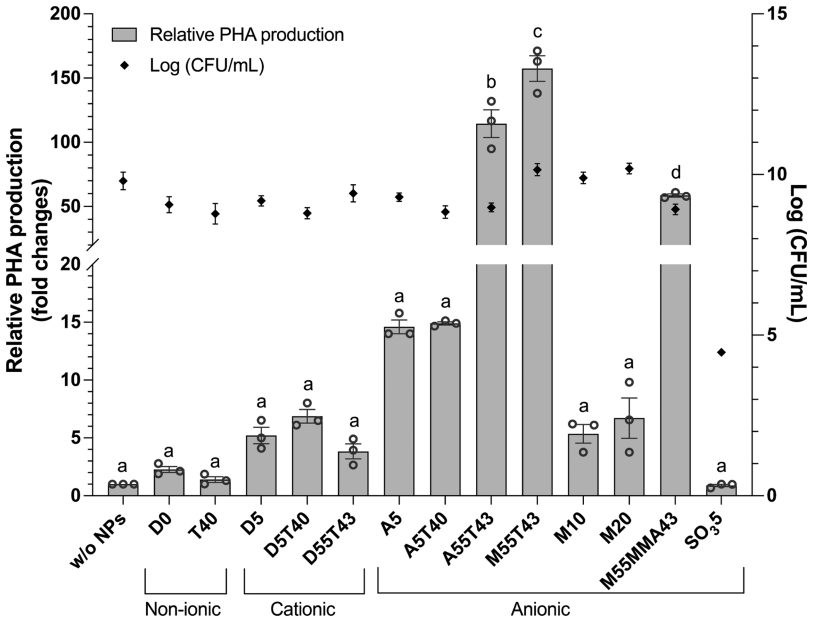

Figure 1. Screening the effect of engineered nanogel particles on the growth and PHA accumulation of *R. sulfidophilum*. The 13 engineered nanogel particles consisted of nonionic, cationic, and anionic nanogel particles, and the composition of each particle is described in Table 1. *R. sulfidophilum* was cocultivated with each of the engineered nanogel particles at a concentration of 1.0 mg/mL under photoautotrophic conditions. Culture without the addition of nanogel particles (w/o NPs) was performed as a control experiment. Viable cells (indicated by black dots) and relative PHA production (indicated by gray bars) were observed at 72 h of culture. The relative PHA production was calculated with respect to the PHA accumulation amount in the control experiment (w/o NPs, without nanogel particles), the absolute PHA values were presented in Table S3. Cultivation was performed in three biological replicates (n = 3), and the error bars represent the standard error of the mean (S.E.). Different letters indicate significant differences in PHA concentration with P values < 0.05 (n = 3 independent biological replicates).

light-emitting diode (LED) light (730 nm, ~20 W m^{-2}, VBP-L24-C3, Valore, Tokyo, Japan) at 30 °C under static conditions until the optical cell density at an absorbance of 660 nm (OD_{660}) reached ~1−1.5 (mid-log phase). Then, the culture was transferred to the C-free M6 medium (M6 medium without sodium malate) supplemented with 20 mM $NaHCO_3$ and 8 mM sodium thiosulfate. The cell density was adjusted to OD_{660} = 0.1 and maintained under the same conditions as mentioned above until the culture reached the mid-log phase (OD_{660} ~ 0.5, Figure S2).

For the photoautotrophic culture of *R. sulfidophilum* with nanogel particles, the seed culture was inoculated into the C-free M6 medium supplemented with 20 mM $NaHCO_3$. The initial culture was standardized to OD_{660} = 0.1, which is equal to 6.5 Log (CFU/mL) (Figure S2) in a sterile screw-cap vial with a silicone septum. UV-sterile nanogel particles were added at a concentration range of 0.1−2 mg/mL. Then, the culture was bubbled with N_2 gas for 5−10 min and maintained under the same culture conditions as mentioned above.

***R. sulfidophilum* Growth Profile.** The time−course profile of *R. sulfidophilum* cell growth was observed at 24 intervals using the plate count technique. To obtain the number of colony-forming units (CFUs), the culture broth was diluted by performing serial dilution; 100 μL of the culture broth was diluted into 900 μL of the C-free M6 medium. Next, 100 μL of the mixture was mixed with 900 μL of the C-free M6 medium. This procedure was repeated to obtain the appropriate cell number for plate counting. Then, 100 μL of the diluted culture broth was spread on a marine agar (BD Difco, New Jersey, USA) plate and incubated at 30 °C for 48−60 h under continuous far-red LED light. The number of colonies on the plate was counted, and the number of CFUs per milliliter was calculated by multiplying by the dilution factor and reported as Log (CFU/mL).

PHA Quantification. Cultures at days 0, 3, 4, 5, and 7 were harvested by centrifugation at 8000*g* for 10 min at 4 °C, washed with distilled water twice, kept at −80 °C overnight, and then freeze-dried for 24−48 h. The PHA content characterization method followed a method previously described.[10,11,23−25] The freeze-dried cells were subjected to methanolysis [1:1 chloroform and methanolysis solution (methanol/sulfuric acid, 85:15 v/v)] at 100 °C for 180 min. After cooling to room temperature, phosphate buffer (pH 8) was added to the reaction mixture and neutralized with 1 N NaOH. The chloroform layer was transferred to a glass tube containing sodium sulfate (anhydrous) to remove the contaminated water.

The PHA content was determined using a chromatography−mass spectrometry (GC−MS) instrument (GCMS-QP2010 Ultra, Shimadzu, Kyoto, Japan) equipped with a 30 m × 0.25 mm × 0.25 μm DB-1 capillary gas chromatography column (Agilent Technologies, CA, USA). For analysis, the injection temperature was set at 250 °C with splitless injection, and the interface temperature was 230 °C. The column temperature program used for ethyl ester separation was as follows: 45 °C for 1 min, a temperature ramp of 7 °C per min to 117 °C. The carrier gas was helium with a flow rate of 3.30 mL min^{-1}. The PHA content was quantified using a standard calibration curve.

Isotopic Analysis of Proteinogenic Amino Acids from the ^{13}C Tracer Experiment. The ^{13}C tracer experiment was carried out with [^{13}C]$NaHCO_3$ (purity of 99 atom %; Cambridge Isotope Laboratories, Massachusetts, USA). During photoautotrophic growth, the C-free M6 medium was supplemented with 20 mM [^{13}C]-$NaHCO_3$. Mid-log-phase seed culture was used to inoculate the C-free M6 medium with the labeled substrate to an initial OD_{660} = 0.1. UV-sterile nanogel particles (M55T43 or A55T43) were added at a concentration of 1 mg/mL, and the culture was bubbled with N_2 gas for 5−10 min. The culture was maintained under continuous far-red LED light (730 nm, ~20 Wm^{-2}) at 30 °C under static conditions. The culture was prepared in duplicate. Samples were harvested at two-time points at the initial time (within 10 min after the addition of [^{13}C]$NaHCO_3$) and the end of the exponential growth phase (72 h of culture). The cells were collected in a volume equivalent to OD_{660} = 3, pelleted by centrifugation at 8000*g* for 10 min at 4 °C, washed twice with distilled water, and kept at −80 °C until analysis. The isotopic analysis of proteinogenic amino acids was performed using GC−MS as previously described.[26,27]

Statistical Analysis. The comparisons of means between different groups were performed using one-way analysis of variance (ANOVA),

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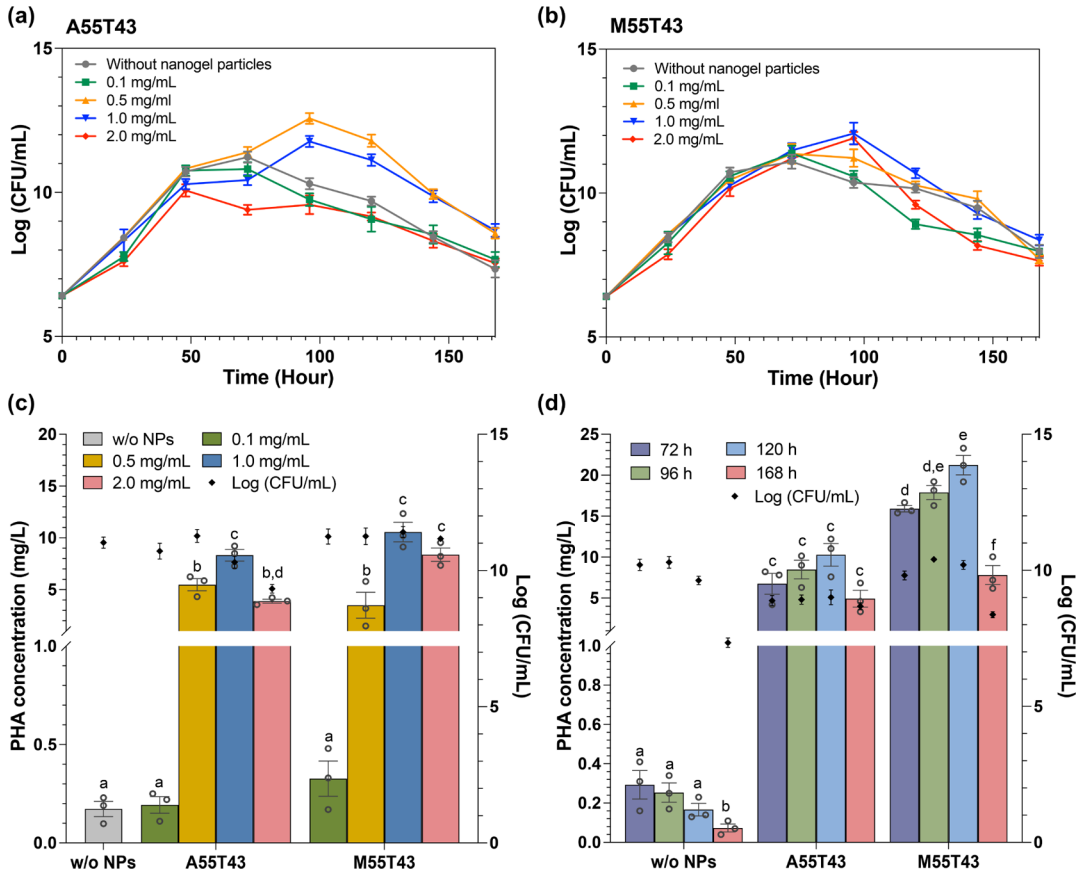

Figure 2. CFU profile (a,b) and PHA production (c,d) of *R. sulfidophilum* cultivated in the presence of nanogel particles (A55T43 or M55T43) under photoautotrophic cultivation. (a,b) *R. sulfidophilum* CFU profile after the addition of A55T43 (a) or M55T43 (b) nanogel particles at various concentrations (0.1, 0.5, 1.0, and 2.0 mg/mL) over 168 h. The initial culture (at 0 h) was standardized to an optical density (OD_{660}) of 0.1, which was equal to 6.5 Log (CFU/mL) (Figure S2). (c) The PHA production after 72 h of photoautotrophic cultivation under different nanogel particle concentrations (range of 0.1−2 mg/mL) is shown in the bar graph. The values in Log (CFU/mL) indicate the viable cell numbers at the time of harvest and are shown as dots. (d) PHA production after 72 h (purple bar), 96 h (green bar), 120 h (blue bar), and 168 h (pink bar) under photoautotrophic cultivation with the addition of 1 mg/mL nanogel particles. The dots indicate the viable cell number in Log (CFU/mL) at the time of harvest. Cultivation was conducted at 30 °C under static conditions under a continuous light supply using LED-produced 730 nm far-red light. Cultivation was performed in three biological replicates (n = 3), and the error bars represent S.E. values. Different letters indicate significant differences in PHA concentration with P values < 0.05 (n = 3 independent biological replicates).

followed by Tukey's multiple comparison test using a statistical significance level of $P < 0.05$. All statistical analyses were carried out using GraphPad Prism version 9.1.0 for macOS (GraphPad Software, San Diego, California USA). The adjusted P values for Figures 1, 2c,d, and 4b are available in Tables S8−S12.

RESULTS AND DISCUSSION

Screening the Effect of 13 Engineered Nanogel Particles on *R. sulfidophilum* Culture. *R. sulfidophilum* is well known for its metabolic versatility, which allows it to grow under either photoheterotrophic or photoautotrophic conditions.[12] To evaluate the effect of engineered nanogel particle addition on photoautotrophic growth, we cultured *R. sulfidophilum* with $NaHCO_3$ supplemented as a sole carbon source with/without the addition of engineered nanogel particles under batch culture conditions. Thirteen engineered nanogel particles, consisting of nonionic-type (D0 and T40), cationic-type (D5, D5T40, and D55T43), and anionic-type nanogel particles (A5, A5T40, A55T43, M55T43, M10, M20, M55MMA43, and $SO_3$5), were prepared via free-radical polymerization[20,21] and evaluated at a concentration of 1.0 mg/mL. Here, D, T, A, M, MMA, and $SO_3$5 stands for *N*-3-[(dimethylamino)propyl]methacrylamide [(DMAPM) amine-containing monomer], TBAm (hydrophobic monomer), acrylic acid [(AAc) carboxylic acid-containing monomer], methacrylic acid [(MAc) carboxylic acid-containing monomer], MMA; hydrophobic monomer, and 2-acrylamido-2-methyl-1-propanesulfonic acid [(AMPS) sulfonic acid-containing monomer], respectively. The monomer compositions of all nanogel particles are described in Tables 1 and S1.[21,22] The culture conditions after adding nanogel particles were controlled at pH ∼7 and 30 °C (Table S2), which are the optimum growing conditions for *R. sulfidophilum*. The effect on cell growth was observed using viable plate counting instead of the optical cell density using the absorbance at 660 nm (OD_{660}) to avoid absorbance interference from nanogel particle addition.

Based on the CFU number observed at 72 h of cocultivation, only $SO_3$5 nanogel particles (anionic type containing sulfonic acids) drastically decreased cell growth compared to the control experiment (without nanogel particles, w/o NPs), with values of 4.47 Log (CFU/mL) and 9.80 Log (CFU/mL), respectively (Figure 1 and Table S3). The inhibition effect may occur due to the strong acid (sulfonic acid groups) in AMPS, monomer composing the $SO_3$5 particles, which possess

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antimicrobial properties.[28,29] However, none of the other 12 nanogel particles, including anionic-type particles containing carboxylic acids, negatively affected *R. sulfidophilum* cell growth (Figure 1 and Tables S3 and S7).

We further analyzed the effect of nanogel particle addition on PHA accumulation in *R. sulfidophilum* under photoautotrophic cultivation because the bacterium naturally synthesizes PHA for nutrient storage and mainly accumulates PHA in the form of poly[(*R*)-3 hydroxybutyrate].[11] Therefore, PHA mentioned in this study is referred to as poly[(*R*)-3 hydroxybutyrate]. PHA was extracted from *R. sulfidophilum* cell pellets harvested at 72 h after cocultivation, and the quantity of PHA was estimated using GC−MS. Surprisingly, the addition of the nanogel particles containing 55 mol % of carboxylic acid (A55T43, M55T43, and M55MMA43) increased PHA accumulation 114-, 157-, and 58-fold, respectively, compared to the control (w/o NPs) (Figure 1, the absolute PHA values are presented in Table S3). The confocal laser scanning microscopy (CLSM) analysis confirmed that PHA was accumulated as an intracellular granule in *R. sulfidophilum* (Figure S1). The nanogel particles with 5, 10, and 20 mol % of carboxylic acids (A5, A5, T40, M10, and M20) and the nanogel particles with 5 and 55 mol % of DMAPM (D5, T40, and D55T43) did not significantly increase the PHA accumulation. These results suggested that the engineered nanogel particles containing a large number of carboxylic acids (A55T43, M55T43, and M55MMA43) can be applied in cocultivation to enhance PHA accumulation by *R. sulfidophilum* under photoautotrophic cultivation.

Growth and PHA Accumulation of *R. sulfidophilum* under Photoautotrophic Cultivations with the Addition of A55T43 and M55T43 Nanogel Particles. We further evaluated the effect of A55T43 and M55T43 nanogel particle addition on photoautotrophic growth. *R. sulfidophilum* was cultivated under $NaHCO_3$ supplemented as a sole carbon source with/without the addition of various concentrations (0.1−2.0 mg/mL) of nanogel particles in the batch culture. Cell growth was monitored over time using viable plate counting.

Based on the time−course profile of the CFU number, the addition of A55T43 nanogel particles in the range of 0.1−1.0 mg/mL showed a gradual increase in the CFU number with a trend similar to that of the control (w/o NPs) for the initial 48 h; after that, the CFU number was stable at approximately 10.72−11.22 Log (CFU/mL) until 72 h (Figure 2a). After that, a gradual decrease in the CFU number was observed under the addition of nanogel particles in the concentration range of 0−0.1 mg/mL, most likely caused by the loss of the carbon source over the long culturing period. Although a relatively low CFU number was observed, a similar growth pattern was obtained with 2.0 mg/mL A55T43 nanogel particles. Interestingly, from 72 to 96 h, the CFU number increased from 11.40 and 10.50 to 12.57 and 11.77 Log (CFU/mL) under the addition of A55T43 nanogel particles at 0.5 and 1.0 mg/mL, respectively, and gradually decreased afterward (Figure 2a).

With the addition of M55T43 nanogel particles, the CFU profile of *R. sulfidophilum* gradually increased in a similar trend for all tested concentrations (0.1−2.0 mg/mL) compared to the control (w/o NPs) until 72 h. Interestingly, the CFU number continued to increase after 72 h and reached approximately 12.07 Log (CFU/mL) at 96 h in the cultures containing 1.0 and 2.0 mg/mL. A similar growth pattern was observed in the control (w/o NPs) and under nanogel particle addition at a concentration of 0.1 mg/mL, in which the CFU number increased continuously, reached approximately 11.09−11.39 log CFU/mL at 72 h, and then gradually decreased. At a concentration of 0.5 mg/mL, the CFU number increased until reaching 11.37 log CFU/mL at 72 h, remained stable until 96 h, and then gradually decreased.

We analyzed PHA accumulation in *R. sulfidophilum* grown under photoautotrophic cultivations containing various concentrations of A55T43 and M55T43 nanogel particles at 72 h after cocultivation. A drastic increase in PHA accumulation was observed with the addition of both nanogel particles at concentrations of 0.5, 1.0, and 2.0 mg/mL compared to the control (w/o NPs) (Figure 2c). The highest accumulation of PHA was observed at a concentration of 1.0 mg/mL with both A55T43 and M55T43 nanogel particles, yielding 8.33 ± 0.56 and 10.57 ± 0.95 mg/L PHA, respectively (Figure 2c and Table S4).

We then examined PHA accumulation in prolonged batch culture containing A55T43 and M55T43 nanogel particles at a concentration of 1.0 mg/mL for 168 h. *R. sulfidophilum* cells were sampled for PHA measurement at 72, 96, 120, and 168 h of cocultivation. In the control experiment (w/o NPs), PHA accumulation reached the highest concentration at 72 h of cultivation (0.29 ± 0.07 mg/L) and decreased gradually until the end of culture (168 h, 0.07 ± 0.02 mg/L) (Figure 2d and Table S5). In contrast, PHA accumulation continuously increased and reached the highest concentration at 120 h of cultivation in the presence of either A55T43 or M55T43, with concentrations of 10.28 ± 1.38 and 21.23 ± 1.18 mg/L, respectively (Figure 2d and Table S5).

To confirm that the particle form is necessary for PHA enhancement and eliminate the possibility that nanogel particles were digested and assimilated in *R. sulfidophilum* culture, we examined the growth and PHA accumulation of *R. sulfidophilum* under culture with the addition of the monomers composed of A55T43 and M55T43 nanogel particles (the results are shown in Supporting Information, Figures S3 and S4). Each of the monomers (TBAm, Bis, AAc, and MAc) and the polymer form of AAc and MAc (PAAc and PMAc) were supplemented to *R. sulfidophilum* culture. The *R. sulfidophilum* viable cell profiles were observed for 168 h (Figure S3). The lower growth profile of *R. sulfidophilum* was observed in the culture with the addition of the monomers composed of A55T43 and M55T43 compared to the control ($NaHCO_3$ addition, w/o NPs), and significantly decreased cell growth was observed in the culture with the addition of AAc and MAc monomers (Figure S3). A growth pattern comparable to that of the control was observed only in the culture with the addition of A55T43 and M55T43 nanogel particles (Figure S3). We then observed PHA accumulation in the culture with the addition of the monomers composed of A55T43 and M55T43. Only the culture with the addition of A55T43 and M55T43 nanogel particles was found to greatly enhance PHA accumulation in *R. sulfidophilum* (Figure S4). To confirm the ability of *R. sulfidophilum* to utilize these monomers as a carbon source to support its growth, we then tested the growth of *R. sulfidophilum* with the addition of the monomers composed of A55T43 and M55T43 without $NaHCO_3$ supplementation (Figure S4). The viable cell number (Log CFU/mL) did not increase after 72 h of cultivation, suggesting that *R. sulfidophilum* might not utilize monomers composed of A55T43 and M55T43 as the carbon source to support its

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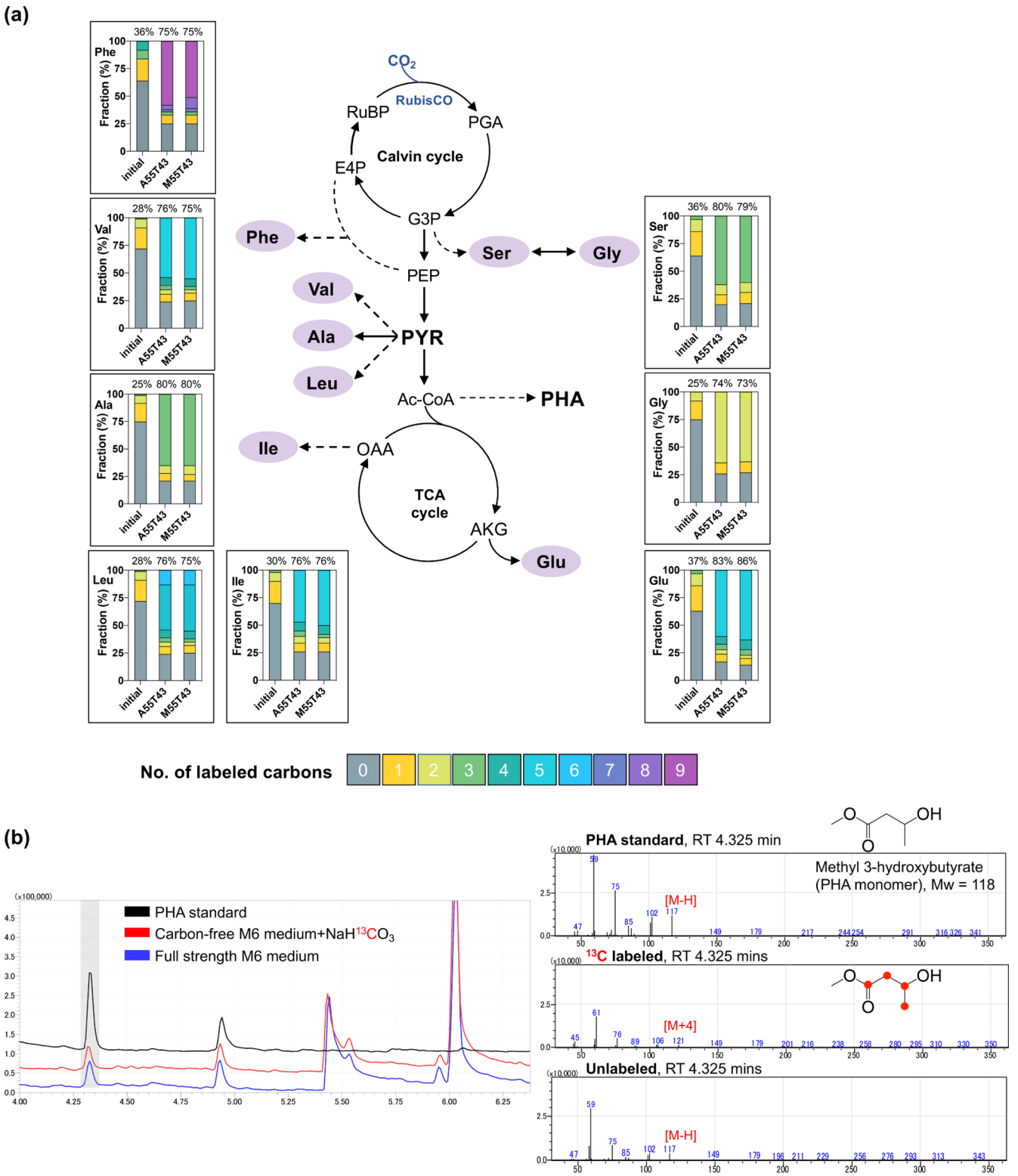

Figure 3. ^{13}C-labeling experiments confirmed the assimilation of HCO_3^- in *R. sulfidophilum*. (a) ^{13}C-Labeling of proteinogenic amino acids confirmed the assimilation of HCO_3^- throughout the central carbon metabolism of *R. sulfidophilum*. *R. sulfidophilum* was cultivated in the presence of nanogel particles (A55T43 or M55T43) under photoautotrophic cultivation. The data from the labeling experiments are averages from duplicates harvested at the end of the exponential growth phase (72 h of culture). Solid-line arrows represent a single-step pathway; dashed-line arrows represent a multiple-step pathway. Abbreviations: RuBP, ribulose 1,5-bisphosphate; PGA, 3-phosphoglycerate; G3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; Ac-CoA, acetyl-Co A; AKG, α-ketoglutarate; OAA, oxaloacetate; PHA, polyhydroxyalkanoate; Phe, phenylalanine; Val, valine; Ala, alanine; Ile, isoleucine; Ser, serine; Gly, glycine; and Glu, glutamate. (b) GC−MS analysis of ^{13}C-labeled PHA compared to that of unlabeled and standard PHA. The full ^{13}C-labeled pattern of 3-hydroxybutyric acid (PHA monomer) was detected, confirming the incorporation of HCO_3^- into accumulated PHA in *R. sulfidophilum*.

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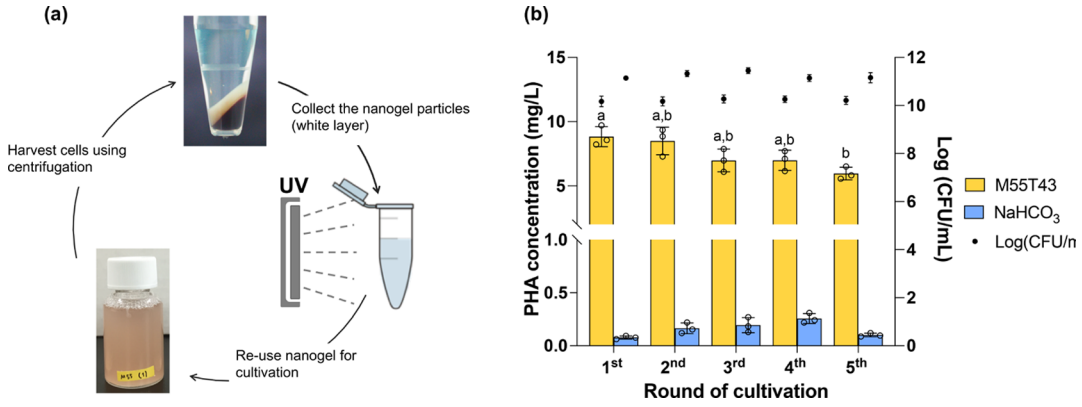

Figure 4. Reusability of M55T43 nanogel particles for *R. sulfidophilum* culture. (a) The M55T43 nanogel particles were separated from the culture broth using centrifugation. The nanogel particle layer was collected and subjected to UV sterilization before the next cycle of *R. sulfidophilum* cultivation. (b) PHA production after 72 h of photoautotrophic cultivation under five rounds of repeated use of M55T43 nanogel particles, as shown by the yellow bars. *R. sulfidophilum* cultivated under $NaHCO_3$ without nanogel particle addition was used as a control in each round (blue bars). The dots indicate the viable cell number in Log (CFU/mL) at the time of harvest. Cultivation was performed in three biological replicates (n = 3), and the error bars represent S.E. values.

growth (Figure S4). These results not only suggested that the nanoparticle forms of A55T43 and M55T43 are crucial for growth support and PHA enhancement in *R. sulfidophilum* under photoautotrophic cultivation but also eliminated the possibility that the engineered nanogel particles can be digested and assimilated as a carbon supply for *R. sulfidophilum*.

^{13}C Labeling of Proteinogenic Amino Acids Confirmed the Assimilation of HCO_3^- Throughout the Central Carbon Metabolism of *R. sulfidophilum*. We then confirmed the assimilation of bicarbonate anion (HCO_3^-) into the metabolism of *R. sulfidophilum* cultivated in the presence of either A55T43 or M55T43 nanogel particles. The ^{13}C tracer experiment was carried out using [^{13}C]$NaHCO_3$ as the sole carbon source in the culture. The culture was harvested at the end of the exponential growth phase (72 h of culture) for the labeling analysis of proteinogenic amino acids. The fragmentation profiles of 14 proteinogenic amino acids were analyzed, and their ^{13}C-labeling degrees were evaluated.

We further mapped the amino acid profile to the precursor metabolites in the central carbon metabolism (Figure S5). Amino acid fragments produced by the same precursors showed similar labeling patterns across different substrates (Figure S5). Similar profiles were obtained from the cultures in the presence of either A55T43 or M55T43 nanogel particles (Figures 3a and S5). More than 70% of all 14 proteinogenic amino acids were labeled by ^{13}C (Figures 3a and S5), indicating that HCO_3^- was assimilated throughout the central carbon metabolism of *R. sulfidophilum*. The results confirmed that the metabolites in *R. sulfidophilum* metabolism were mainly incorporated from HCO_3^-, suggesting that the accumulated PHA was most likely also incorporated from HCO_3^-.

To provide evidence that the accumulated PHA was indeed incorporated from HCO_3^-, we further conducted GC−MS analysis of PHA obtained from *R. sulfidophilum* cultivated under [^{13}C]$NaHCO_3$ as a sole carbon source with the addition of M55T43 nanogel particles. The results showed a peak at the same retention time as that corresponding to poly-3-hydroxybutyrate (Figure 3b). The mass fragmentation pattern of the peak corresponding to methyl 3-hydroxybutyrate (PHA monomer) showed the full ^{13}C-labeled pattern (Figure 3b), indicating $H^{13}CO_3^-$ incorporation into the accumulated PHA.

Reusability of M55T43 Nanogel Particles for *R. sulfidophilum* Cultivation. The M55T43 nanogel particles could be separated and collected after cultivation using centrifugation (Figure 4a). Thus, we questioned whether these nanogel particles could be reused for the repeated cultivation of *R. sulfidophilum*. Used nanogel particles were separated from the cell pellet and subjected to UV sterilization before being added to the next cycle of *R. sulfidophilum* cultivation (Figure 4a). Cultivation was conducted using $NaHCO_3$ as the sole carbon source.

PHA accumulation in *R. sulfidophilum* cells was observed in five cultivation cycles with the repeated use of M55T43 nanogel particles. As expected, the reused M55T43 nanogel particles maintained PHA-enhancing performance for at least five repeated cultivation cycles. Variations in PHA concentration in the range of 5.96−8.83 mg/L were observed in each cultivation cycle (Figure 4b and Table S6). We noticed a reduction in PHA accumulation after each cultivation cycle. An approximately 32.50% reduction between the first and fifth rounds of cultivation was observed (Figure 4b). This reduction might have been caused by the loss of M55T43 nanogel particles during the collection step after each cultivation cycle. The recovery efficiency of M55T43 nanogel particles was observed by measuring their dried weight at the beginning of the first round compared to the dried weight at the end of the fifth round of cultivation, and about 61.1% recovery was observed (Table S7).

Effects of the Engineered Nanogel Particles. Here, we aimed to demonstrate the application of the engineered nanogel particles for microbial bioproduction. We used *R. sulfidophilum*, a marine photosynthetic bacterium capable of growing under either photoheterotrophic or photoautotrophic conditions.[12] This bacterium has been proposed as a promising cell factory for sustainable bioproduction, including as a host for PHA production.[13] Although *R. sulfidophilum* has been confirmed to synthesize PHA naturally, the use of organic carbon sources, such as malate, acetate, or pyruvate, is required.[14,15] In this study, in an attempt to produce PHA under photoautotrophic cultivation, we applied engineered nanogel particles to an *R. sulfidophilum* photoautotrophic cell

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factory. By employing this technique, we successfully enhanced PHA accumulation in *R. sulfidophilum* grown under photoautotrophic cultivation.

We screened the effect of 13 engineered nanogel particles on the cell growth and PHA accumulation of *R. sulfidophilum*. The screening results narrowed the field down to three nanogel particles that contain a large number of carboxylic acids (A55T43, M55T43, and M55MMA43) due to their ability to support the growth and enhance PHA accumulation under photoautotrophic cultivation (Figure 1). We then observed the CFU profile of *R. sulfidophilum* in the presence of A55T43 and M55T43 in photoautotrophic cultivation. In the cultures containing A55T43 at concentrations of 0.5 and 1.0 mg/mL, the log phase was observed until 48 h. However, the CFU number increased again from 72 to 96 h (Figure 2a), while the PHA accumulation was not significantly increased during this period (Figure 2d), which may reflect the use of accumulated PHA to support growth after a lack of nutrients. In the cultures containing M55T43 at 1.0 and 2.0 mg/mL concentrations, a prolonged log phase was observed until 96 h (Figure 2b). We speculated that this prolonged log phase might benefit PHA accumulation in *R. sulfidophilum* because PHA mostly accumulates during the log phase of microbial growth.[25,30,31] The highest PHA accumulation was observed at 21.23 ± 1.18 mg/L in the culture containing M55T43 at 120 h of cultivation (Figure 2d and Table S5).

To eliminate the possibility that the monomers of nanogel particles were digested and assimilated by *R. sulfidophilum*, leading to the enhancement of PHA accumulation, we supplemented each monomer composed of A55T43 and M55T43 in *R. sulfidophilum* cultures (the results are shown in Figures S3 and S4). By observing the viable cell number and PHA accumulation of *R. sulfidophilum* with the supplementation of monomers composed of A55T43 and M55T43, we confirmed that the monomers were not the reason for high PHA accumulation in *R. sulfidophilum* (Figure S4). To ensure HCO_3^- utilization by *R. sulfidophilum* in the cultures containing nanogel particles, we employed a ^{13}C tracer experiment. The obtained results showed that all tested proteinogenic amino acids were labeled by ^{13}C isotopes (Figures 3a and S5), which indicated that HCO_3^- was assimilated throughout the central carbon metabolism, suggesting its incorporation into the accumulated PHA. Indeed, the GC−MS mass fragmentation pattern of extracted PHA from the *R. sulfidophilum* culture containing nanogel particles confirmed the incorporation of $[^{13}C]NaHCO_3$ into PHA accumulated in *R. sulfidophilum* (Figure 3b).

Considering the monomer compositions and chemical structures of the nanogel particles, only three nanogel particles (A55T43, M55T43, and M55MMA43) of the anionic type, especially A55T43 and M55T43, correlated with high PHA accumulation. A55T43 and M55T43 nanogel particles share a similar chemical structure in which TBAm constructs a hydrophobic microenvironment in the particles, while AAc and MAc are mostly located in the hydrophobic microenvironment, according to previous studies.[21,22] The surface zeta potential of the engineered nanogel particles is shown in Table S1. A55T43 and M44T43 possess negative values (−40.4 and −49.2 mV, respectively), suggesting that they are stable in the colloidal system. The surface zeta potential of *R. sulfidophilum* (−39.3 mV) was similar to that of A55T43 and M44T43, suggesting that they are unlikely to get attached to the bacterial cell surface. This might also be the reason why the engineered nanogel particle (M55T43) can be recovered by simple centrifugation after the cocultivation (Figure 4A). Even though the mechanism of how the engineered nanogel particles impact the PHA accumulation in *R. sulfidophilum* cell factory was not clarified yet, we speculated that the weak acidic groups in the particles of A55T43 and M55T43 nanogel particles, together with their ability to capture salt ions,[22] may create a stress microenvironment inside the *R. sulfidophilum* culture. A previous report demonstrated that the accumulation of PHA plays an essential role in the salt and pH tolerance of microorganisms.[32−35] Therefore, the stress microenvironment created by nanogel particle addition may enhance PHA accumulation in *R. sulfidophilum* under photoautotrophic cultivation. However, a series of deeper analyses would be required to reveal the mechanism of the engineered nanogel particle's impact on *R. sulfidophilum* cell factory.

Reusability performance is one of the necessary criteria for nanoparticles to be applied to sustainable bioproduction.[36] In this matter, we observed that the used M55T43 nanogel particles could be separated after cultivation using centrifugation. Our evaluation of the reusability of M55T43 showed that it could maintain PHA-enhancing performance for at least five repeated cycles of *R. sulfidophilum* cultivation (Figure 4b). Although a reduction in accumulated PHA was observed in each cultivation cycle, we assumed that this reduction might be caused by the loss of nanogel particles during the collection step. Therefore, an efficient method to collect used nanogel particles after every cultivation cycle would benefit practical usage.

CONCLUSIONS

This study demonstrated the application of engineered nanogel particles for bioproduction in a microbial cell factory. By simply supplementing cultures with the engineered nanogel particles, we successfully enhanced PHA accumulation in *R. sulfidophilum* under photoautotrophic conditions up to 157-fold using $NaHCO_3$ as the sole carbon source. Using a series of experiments, we confirmed that the accumulated PHA was indeed incorporated from $NaHCO_3$. Furthermore, the nanogel particles could maintain PHA-enhancing performance for at least five cycles of *R. sulfidophilum* cultivation. Indeed, our near-future goal is the direct assimilation of CO_2 and N_2 as the sole carbon and nitrogen source for photoautotrophic PHA production in *R. sulfidophilum*, which may require genetic modification to achieve. However, our results indicate the success of using engineered nanogel particles to enhance PHA accumulation in the *R. sulfidophilum* cell factory, bringing us closer to sustainably producing PHA under photoautotrophic conditions. The strategy of using the engineered nanogel particles will complement the other strategies (such as genetic modification to engineer metabolic pathways) to synergistically enhance the biorefinery efficiency, which may be applied to other microbial cell factories to produce other commodity chemicals.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.1c07252.

Composition and physical properties of nanogel particles used in this study; detailed information of absolute PHA concentration and viable cell number of *R. sulfidophilum*

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cocultivated with nanogel particles under photoautotrophic conditions; recovery efficiency of an engineered nanogel particle; effect of monomer compositions of engineered nanogel particles on PHA accumulation in *R. sulfidophilum*; and mapping of the ^{13}C-labeled proteinogenic amino acid profile (PDF)

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Author Contributions

K.N. conceptualized the research. P.S. and M.H.-T. performed the photosynthetic bacteria-related experiments. R.H. and Y.H. synthesized the engineered nanogel particles. T.S. and A.K. were involved in the isotopic analysis of proteinogenic amino acids. P.S., M.H.-T., and K.N. analyzed the data. P.S. wrote the manuscript. All the authors revised the manuscript.

Notes

The authors declare no competing financial interest.

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