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Advanced functionalization of polyhydroxyalkanoate via the UV-initiated thiol-ene click reaction

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Abstract

Polyhydroxyalkanoates (PHAs) incorporating vinyl-bearing 3-hydroxyalkanoates were prepared in 8.5–12.9 $g \cdot L^{-1}$ yield. The molar ratios (0–16 mol%) of the vinyl-bearing 3-hydroxyalkanoate derivatives were controlled by the continuous feeding of undecylenate at various concentrations. Subsequently, the PHAs were functionalized by UV-initiated thiol-ene click reaction and chemical modification. ^{1}H NMR spectra suggested that 3-mercaptopropionic acid and 2-aminoethanthiol were successfully introduced into the vinyl-bearing PHA. Subsequently, chemical modification using fluorescein or a fibronectin active fragment (GRGDS) was attempted. The former yielded a PHA derivative capable of emitting fluorescence under UV irradiation, which was useful for determining the miscibility of PHA in a composite film comprising poly-L-lactic acid (PLLA) and PHA. In the latter case, PHA bearing GRGDS peptides exhibited cell adhesiveness, suggesting that its biocompatibility was improved upon peptide introduction. Taken together, the UV-initiated thiol-ene click reaction was demonstrated to be useful in PHA modification.

Keywords: polyhydroxyalkanoate, medium chain length, click chemistry, double bond, ene-thiol click reaction

Introduction

Polyhydroxyalkanoates (PHAs) are a class of aliphatic polyesters that accumulate in various bacteria under nutrient-limited conditions, acting as carbon and energy storage materials (Chen 2009; Sudesh et al. 2000). To date, PHAs have attracted much attention, as they possess properties similar to common thermoplastics. PHAs are generally produced by in vivo methods, using whole bacterial cells. They are advantageous in that they can also be produced from renewable resources such as sugars and plant oils, and are degraded in the natural environment. However, the applications of poly(3-hydroxybutyrate) [P(3HB)], a common PHA produced by bacteria, are limited because of its brittle and stiff nature, and its low functionality, which depend on the PHA monomer compositions.

Monomer compositions of PHAs are mainly determined by the substrate specificities of PHA synthases. PHA synthases, which are key enzymes in PHA biosynthesis, catalyze the polymerization of (*R*)-hydroxyalkanoyl moieties in (*R*)-hydroxyalkanoyl coenzyme A (HACoA), with the concomitant release of CoA (Rehm 2003; Taguchi and Doi 2004). The HACoA supply pathway to PHA synthases also affects the monomer compositions depending largely on cell metabolic pathways, e.g., de novo fatty acid biosynthesis, its degradation pathways, and on substrate feedings.

Copolymerization with various hydroxyalkanoate (HA) units is an effective method to improve the physical properties and functionalities of PHA, and in this respect, the syntheses of various copolymers have been attempted (Dai et al. 2008; Fukui et al. 1999; Han et al. 2009; Hu et al. 2011; Matsumoto et al. 2011; Matsusaki et al. 1998; Nomura et al. 2004; Page et al. 1992; Shozui et al. 2009; Tajima et al. 2009; Tajima et al. 2012; Tanadchangsaeng et al. 2009; Valentin et al. 2000; Valentin, Reister and Gruys 2000). Recently, the in vivo synthesis of PHA incorporating lactate (LA) was achieved in a single step through the introduction of genes encoding the screened PHA synthase (lactate-polymerizing enzyme) into *E. coli*

engineered to accumulate LA-CoA in the cell (Fig. 1, Method 1) (Matsumoto and Taguchi 2010; Shozui et al. 2010; Taguchi et al. 2008; Yamada et al. 2010).

As an alternative approach, we focused our attention on the production of novel PHAs via chemical modification using a click reaction to expand the application range of PHAs (Fig. 1, Method 2). Click reactions are generally high yielding, wide in scope, stereospecific, simple, and produce by-products that can be easily removed without chromatography. Such reactions are widely used as emerging synthetic tools for both high- and low-molecular-weight compounds. One representative example of a click reaction is the Huisgen copper(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC), which yields a 1,4-disubstituted five-membered 1,2,3-triazole ring (Kolb et al. 2001). This reaction between azides and alkynes offers high yields and involves functionalities that can be introduced relatively easily in a variety of molecules. In addition to the CuAAc, the thiol-ene reaction is also important in the realm of click chemistry (Carioscia et al. 2007; Hoyle and Bowman 2010, Hoyle et al. 2004; Li et al. 2007). Campos et al. reported the synthesis of various macromolecules via the UV-initiated thiol-ene click reaction, which proceeded quantitatively, with a high degree of specificity in a matter of minutes (Campos et al. 2008; Killops et al. 2008). However, the majority of PHAs studied to date contain no functional groups, and few groups have reported such modification of PHAs (Constantin et al. 1999; Gagnon et al. 1994a, b; Park, Lenz and Goodwin 1998). Thus, to establish a synthetic methodology for novel PHAs, we explore the preparation of PHA bearing vinyl groups (by undecylenate feeding) and their subsequent chemical modifications using a click reaction.

Materials and methods

Materials

Peptone, yeast extract, chloroform, methanol, glycerol, 10-undecenoic acid, potassium dihydrogen phosphate (KH_2PO_4), diammonium hydrogen phosphate [$(NH_4)_2HPO_4$], citric acid, triethylamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Fluorescein isothiocyanate (FITC), chlorobenzene, and acetone were purchased from Sigma-Aldrich Japan (Tokyo, Japan). 3-Mercaptopropionic acid, 2-aminoethanthiol hydrochloride, and 2,2-dimethoxy-2-phenylacetophenon (DMPA) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade or better. Poly-L-lactic acid (PLLA) was also purchased from Sigma-Aldrich Japan. The fibronectin active fragment (Gly-Arg-Gly-Asp-Ser: GRGDS) was the product of Peptide Institute, Inc. (Osaka, Japan).

Analytical instrumentation and methods

Polymer composition and accumulation were analyzed using a GC-2010 capillary GC system (Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector and a Zebron ZB-1 GC capillary column (0.25 mm i.d. × 30 m; Phenomenex Inc., CA, USA). Samples were prepared as follows. Polymer (~10 mg) or dried microbial cells (50 mg) were treated with a solution of methanol (1.7 mL), sulfuric acid (0.3 mL), and chloroform (2 mL) at 100 °C for 140 min to convert the constituents to their methyl esters. Water (1 mL) was then added to the reaction mixture, and the chloroform layer was used for GC analysis.

The ^1H nuclear magnetic resonance (NMR) spectra of the polymers were obtained using a Bruker MSL400 spectrometer (400 MHz; Bruker BioSpin K.K., Yokohama, Japan) at

25 °C and a 90° pulse, with a 4 ms, 3,000 Hz spectral width and a 4 s repetition rate. Chemical shifts were reported in ppm using tetramethylsilane (TMS) as an internal reference.

The molecular weights of the obtained polymers were determined by gel permeation chromatography (GPC) using tandem TSKgel Super HZM-H columns (6.0 mm I.D. × 150 mm; TOSOH, Tokyo, Japan) with chloroform as the eluent. Calibration was performed using polystyrene samples as standards.

Ultraviolet (UV) light irradiation of samples was carried out using a UVP Black Ray UV bench lamp XX-15BLB (UVP, LLC, CA, USA).

Media and microorganisms

Nutrient-rich (NR) medium (10 $g \cdot L^{-1}$ peptone, 2 $g \cdot L^{-1}$ yeast extract, 10 $g \cdot L^{-1}$ meat extract) was used for the preparation of cells for precultivation. MR_Pre medium (22.0 $g \cdot L^{-1}$ KH_2PO_4, 3.0 $g \cdot L^{-1}$ $(NH_4)_2HPO_4$, 0.80 $g \cdot L^{-1}$ citric acid, 20.0 $g \cdot L^{-1}$ glycerol) supplemented with 7.0 $mL \cdot L^{-1}$ magnesium sulfate solution [20 %(*w/v*) $MgSO_4 \cdot 7H_2O$] and 10 $mL \cdot L^{-1}$ trace element solution (Kato et al. 1996) was used for precultivation. *Pseudomonas putida* (*P. putida*) KT2440 (ATCC 47054) was used for PHA production. MR_Acc medium (3.0 $g \cdot L^{-1}$ $(NH_4)_2HPO_4$, 0.8 $g \cdot L^{-1}$ citric acid, 20.0 $g \cdot L^{-1}$ glycerol) supplemented with 7.0 $mL \cdot L^{-1}$ magnesium sulfate solution [20 %(*w/v*) $MgSO_4 \cdot 7H_2O$], 10 $mL \cdot L^{-1}$ trace element solution (Kato et al. 1996), and 6.0 $mL \cdot L^{-1}$ antifoaming agent was used for PHA accumulation. The feed solution contained 1500 g of glycerol and 30 g of $MgSO_4 \cdot 7H_2O$ per 3000 mL of distilled water. *P. putida* was grown at 30 °C in NR medium. 10-Undecenoic acid was neutralized by 5N NaOH solution to prepare undecylenate solution (pH 7.0).

Preparation of PHA bearing vinyl groups

A small portion of frozen cells (*P. putida* KT2440) was placed into NR medium (3 mL) using a platinum loop, and shaking carried out at 30 °C for 16 h. The overnight culture

was then inoculated into NR medium (27 mL), and incubated at 30 °C with shaking at 150 strokes·min^{-1} for 10 h. The culture (14 mL) was then inoculated into MR_Pre medium (700 mL), and incubated at 30 °C with shaking at 140 strokes·min^{-1} for 16 h. The resulting overnight culture was inoculated into MR_Acc medium (3500 mL), and then incubated at 30 °C rotating at 150 rpm for 72 h in a 10 L jar fermenter (BMS-10NP3 DPC-3A; ABLE corporation, Tokyo, Japan). The feed solution (168 mL per shot) containing glycerol and $MgSO_4$ was added to the jar fermenter using the pH-stat method, with an average feeding number of 14. Undecylenate solution was continuously added to the culture from 16 h after beginning the cultivation until the end of cultivation. The final concentrations of undecylenate in the media were 0.15, 0.30, 0.45, and 0.60 %(*w/v*).

The collected bacterial cells were washed twice with ethanol and once with distilled water, and then lyophilized. Polymer was extracted from the dried microbial cell bodies using a Soxhlet extractor with chloroform as solvent, and then precipitated by the addition of methanol after the solvent was reduced with an evaporator under reduced pressure at 30 °C. The structure of the prepared PHA bearing vinyl groups (PHAund60) was analyzed by nuclear magnetic resonance (NMR) spectroscopy (Fig. 2a).

General procedure for the UV-initiated thiol-ene click reaction

The UV-initiated thiol-ene click reaction was performed according to the method described by Campos et al. (Campos et al. 2008). In a vial, the prepared PHA bearing vinyl groups (PHAund60), thiol (10 eq with respect to the vinyl group), and DMPA (0.2 eq) were dissolved in either chlorobenzene or acetone. The vial was sealed with a screw cap, and the solution was purged with argon for 10 min. Irradiation was then applied for 1 h using a 365 nm UV lamp (UVP, LLC, CA, USA). Subsequently, the mixture was washed with chloroform/water to remove impurities, and separated into aqueous and organic phases. The

organic phase was dried over anhydrous $MgSO_4$, and the solvent evaporated to obtain the desired product.

PHAund60-NH_2 (**2a**). 2-Aminoethanethiol and chlorobenzene were used as the thiol compound and solvent, respectively (Fig. 3). In a vial, PHAund60 (**1**, 1.0 g, 5.90 mmol) was dissolved in dried toluene (5 mL), and the solvent was evaporated. This operation was repeated twice to remove water from the starting material. 2-Aminoethanethiol (0.732 g, 9.38 mmol, 10 eq with respect to the vinyl group) was dissolved in chlorobenzene (60 mL) and added to the vial. DMPA (48.0 mg, 0.188 mmol) was then added to the vial, which was subsequently covered with an aluminum foil to block out light. After purging the mixture for 10 min with argon, irradiation with a 365 nm UV lamp (UVP, LLC, CA, USA) was carried out for 1 h. Following purification by precipitation with methanol, introduction of 2-aminoethanethiol to the polymer was confirmed by NMR spectroscopy (Fig. 2b).

PHAund60-COOH (**2b**). 3-Mercaptopropionic acid was used as the thiol compound, and chlorobenzene was employed as the solvent. In addition, PHAund60 (**1**, 1.0 g, 5.90 mmol) and 2-aminoethanethiol (1.06 g, 9.38 mmol, 10 eq with respect to the alkene) were used. Other procedures were the same as that of 2-aminoethanethiol. Following purification, introduction of 3-mercaptopropionic acid to the polymer was confirmed by NMR analysis (Fig. 2c).

Introduction of fluorescein into PHAund60-NH_2

In a vial, PHAund60-NH_2 (**2a**, 1.06 g, 6.24 mmol) was dissolved in dichloromethane (5 mL). Triethylamine (27.4 μL, 1.0 eq with respect to the amino group) was added to the vial and stirred at 30 °C (Fig. 4). FITC (84.9 mg, 1.1 eq with respect to the amino group) was then added, and the resulting mixture was stirred for 2 h. Following this time, the mixture was washed with chloroform/water to remove impurities, and then separated into aqueous and organic phases. The organic phase was dried over anhydrous $MgSO_4$, and the solvent was

evaporated to obtain the desired product. Introduction of fluorescein into PHA was confirmed by fluorescence spectrophotometry.

Introduction of a fibronectin active fragment (GRGDS) into PHAund60-COOH

In a vial, PHAund60-COOH (**2b**, 40 mg, 0.21 mmol) was dissolved in acetone/water (3 mL, 9:1). EDC (6.54 mg, 1.0 eq with respect to the carboxyl group) was added to the vial and stirred at 0 °C for 15 min (Fig. 4). The fibronectin active fragment (GRGDS, 184 mg, 1.1 eq with respect to the carboxyl group) was then added and the resulting mixture stirred at 30 °C for 16 h. Then, the mixture was washed with chloroform/water to remove impurities, and was separated into aqueous and organic phases. The organic phase was dried over anhydrous $MgSO_4$, and the solvent evaporated to obtain the desired product. Introduction of the peptides into PHA was confirmed by NMR analysis (Fig. 2d).

Preparation of a composite film composed of PLLA and PHA with fluorescein

A mixture of PLLA (9.8 g) and PHA bearing fluorescein (**3**, 0.2 g) was dissolved in chloroform (5 mL), and the prepared polymer solution was poured into a PTFE Petri dish. After drying at 30 °C in air for 16 h, the resulting composite film was observed using a fluorescence microscope (BX40; Olympus Corporation, Tokyo, Japan).

Cell cultivation

Human Umbilical Vein Endothelial Cells (HUVEC) were used as cells for evaluating the biocompatibility of PHA bearing GRGDS peptides, because HUVEC has $\alpha_v\beta_3$ integrin that binds to a RGD peptide. HUVEC were cultured in endothelial basal medium-2 (EBM-2; Lonza Group Ltd., Basel, Switzerland) supplemented with EGM-2, a supplement factor set, at 30 °C in air containing 5 vol% CO_2. The culture medium was changed every 3 d. At semi-confluent growth, cells were subcultured in the same medium.

PHAund60-COOH (**2b**: 100 mg) was dissolved in ethanol (15 mL) and poured into a 24-well plate. EDC and the fibronectin active fragment were then added into the wells, which were incubated at 30 °C for 16 h. After air-drying at 30 °C for 24 h, the resulting PHA film was sterilized by UV-irradiation. HUVEC (10,000 cells/well) in EBM-2 medium with EGM-2 were seeded into the 24-wells coated by PHA bearing GRGDS peptides (**4**) and incubated at 37 °C in air containing 5 vol% CO_2. Cells were observed using an inverted microscope (IX40; Olympus Corporation).

Results

Preparation of vinyl-bearing PHAs

A fed-batch cultivation using the pH-stat method was carried out. To produce PHAs bearing vinyl groups of different molar ratios, a solution of undecylenate was added continuously to the medium using a peristaltic pump. The final undecylenate concentrations in the medium were controlled at 0.15–0.60 %(*w/v*). The vinyl molar ratios in the polymers reached a peak of 16 mol%, and were precisely controlled at 0–16 mol% (Fig. 5). The conversion ratios of undecylenate to vinyl-bearing 3HA were approximately 22–40 %. The yields of the PHAs decreased slightly with an increase in undecylenate concentrations (Table 1 and Fig. 5).

The monomer compositions, molecular weights, and dispersities (*D*) of the prepared PHAs are listed in Table 2. The contents of the main PHA components, 3-hydroxyoctanoate (3HO) and 3-hydroxydodecanoate (3HD), decreased with an increase in undecylenate concentration. In addition, the contents of the unsaturated 3-hydroxyalkanoates incorporated into the resulting PHAs (i.e., 3-hydroxy-8-nonenoate (C9:1) and 3-hydroxy-10-undecenoate (C11:1)) also increased in the similar manner (Table 2). Furthermore, when the undecylenate concentration reached 0.60 %(*w/v*), the 3-hydroxy-6-heptenoate (C7:1) unit was detected. The

molecular weights of the prepared PHAs decreased with an increase in undecylenate concentrations, while their dispersities (*D*) were relatively constant at all undecylenate concentrations. The result of NMR analysis of the prepared PHAs suggested that the vinyl groups were successfully incorporated into 3HAs (Fig. 2).

UV-initiated thiol-ene click reaction

Following successful preparation of the vinyl-containing PHAs by fed-batch fermentation, the introduction of amino and carboxyl groups into the PHA was carried out via the UV-initiated thiol-ene click reaction (Fig. 3). PHA (PHAund60) containing ~16 mol% of the vinyl-bearing 3HA was selected as the PHA sample. For introducing amino and carboxyl groups, 2-aminoethanethiol and 3-mercaptopropionic acid were used as thiol compounds, respectively. ^{1}H NMR spectra of the PHAs before and after the click reactions are shown in Fig. 2. Signals corresponding to the vinyl protons were observed at ~4.95 and ~5.80 ppm in the ^{1}H NMR spectrum before reaction (Fig. 2a). After the thiol-ene click reaction using 3-mercaptopropionic acid, these two signals almost completely disappeared (Fig. 2c). In contrast, signals corresponding to the vinyl protons were observed after the reaction with 2-aminoethanethiol (Fig. 2b) likely due to the incomplete reaction. The introduction rates of the two thiol compounds (3-mercaptopropionic acid and 2-aminoethanethiol) as estimated from the ^{1}H NMR spectra were >99 % and 23.0 %, respectively. This confirms that the UV-initiated thiol-ene click reaction is applicable for PHA modification, and in particular the introduction of amino and carboxyl groups.

Introduction of fluorescein into PHAund60-NH_2

The functionalization of PHA through the introduction of fluorescein was performed using PHAund60-NH_2 (Fig. 4), in which the unit molar ratio of amino groups was ~4 mol% [0.16 (unit molar ratio of vinyl groups) × 0.23 (introduction rate of 2-aminoethanethiol) =

0.037]. Following fluorescein introduction into PHAund60-NH_2, green fluorescence was observed upon irradiation with UV light (Fig. 6). The resulting polymer was dissolved in acetone and analyzed by fluorescence spectroscopy. Emission maxima were observed at 480 and 485 nm for the fluorescein-containing PHAund60 and the free FITC, respectively (Fig. 6). Compared to the free FITC, the maximum emission wavelength for fluorescein-PHA was shifted to a shorter wavelength, likely due to a change in the mobility of fluorescein upon conjugation to PHA through the thiourethane linkage.

A number of PLLA-based polymer alloys including PHA have been reported to reduce the brittleness of PLLA. PHAs produced by the *Pseudomonas* species are generally medium-chain-length PHAs (mcl-PHAs) that exhibit high elasticity, and are thus effective in reducing PLLA stiffness. As the prepared fluorescein-PHA is capable of emitting fluorescence upon UV-irradiation, its location can be easily determined through fluorescence observations. Therefore, a composite film composed of PLLA and fluorescein-PHA (**3**) was prepared and observed by fluorescence microscopy. Finely dispersed fluorescein was detected over the entire film (Fig. 7b) in contrast to a composite film composed of PLLA and PHAund60-NH_2 (Fig. 7a), confirming that fluorescein-PHA (**3**) can be used for determining the location of PHA within a composite film.

Introduction of a fibronectin active fragment (GRGDS) into PHAund60-COOH

PHA functionalization through the introduction of a peptide-based bioactive molecule was also performed using PHAund60-COOH (**2b**). As a bioactive molecule, the fibronectin active fragment, a GRGDS peptide, was used to improve the biocompatibility of PHA. By ^{1}H NMR analysis, the peaks corresponding to the peptide were observed around 4 ppm (Fig. 2d).

The condensation reaction was first carried out on the film cast on the bottom of a 24-well plate to ensure that the GRGDS peptides could be easily contacted with the cells (Fig. 4). Following sterilization by UV-irradiation, HUVEC were placed into the wells, and then

incubated at 37 °C under air containing 5 vol% CO_2. The 24-well plate, coated with either GRGDS-PHA or PHAund60-COOH, was monitored with an inverted microscope over 6 h cultivation. HUVEC adhered on the wells were observed for the 24-well plate coated with the PHA bearing GRGDS peptides (Fig. 8), but not for the 24-well plate coated with only PHAund60-COOH (Fig. 8).

Discussion

To date, a number of reports on the use of *Pseudomonas* for the preparation of PHA incorporating unsaturated monomers have been published (Ashby and Foglia 1998; Constantin et al. 1999; Gagnon et al. 1994a, b; Huijberts et al. 1992; Lageveen et al. 198inhi8; Lee et al. 2001; Park, Lenz and Goodwin 1998; Sun et al. 2009). In these studies, various monomers bearing unsaturated units were supplied through β-oxidation and de novo fatty acid synthetic pathways. In this study, undecylenate, which contains a vinyl group, was used as the source of unsaturated monomer to produce 3HAs bearing a vinyl group at the alkyl side chain terminal (Constantin et al. 1999; Gagnon et al. 1994a, b; Park, Lenz and Goodwin 1998). Because of the toxicity of undecylenate to bacteria cells, it was added gradually to the medium using a peristaltic pump. Using such a feeding method, the molar ratios of 3HAs could be precisely controlled at 0–16 mol% (Fig. 5), giving 8.5–12.9 $g \cdot L^{-1}$ vinyl-containing PHA in a single cultivation (Table 1 and Fig. 5).

In general PHA production, the monomer units (3HAs) are supplied via a de novo fatty acid synthetic pathway. In contrast, when undecylenate was added, it appeared that 3HAs bearing vinyl groups were supplied by the β-oxidation pathway based on the following observations: 1) the main components of the PHAs were 3HO and 3HD, and the contents of vinyl-bearing 3HAs decreased with an increase in undecylenate concentration (Table 2); 2) 3-hydroxy-6-heptenoate (C7:1) was also detected in the presence of high undecylenate concentrations (Table 1).

Copolymerization with various hydroxyalkanoate (HA) units is known to be effective in improving the physical properties and functionalities of PHA, and therefore, the syntheses of various copolymers have been attempted (Dai et al. 2008; Fukui et al. 1999; Han et al. 2009; Hu et al. 2011; Matsumoto et al. 2011; Matsusaki et al. 1998; Nomura et al. 2004; Page et al. 1992; Shozui et al. 2009; Tajima et al. 2009; Tajima et al. 2012; Tanadchangsaeng et al. 2009; Valentin et al. 2000; Valentin, Reister and Gruys 2000). However, few studies have been carried out into the introduction of monomer units bearing amino and carboxyl groups, which exhibit high reactivity and are useful for PHA functionalization. In this study, the amino and carboxyl groups were successfully introduced via a UV-initiated thiol-ene click reaction. The introduction rates of amino and carboxyl groups as estimated from the ^{1}H NMR spectra were >99 % and 23.0 %, respectively (Figs. 2 C and B). Although the introduction rate of amino groups was rather low, the introduced amino groups exhibited high reactivity, and the unit molar ratio (~4 mol%) was sufficient for introducing the fluorescent group into PHA (Figs. 6 and 7).

PHAs composed of monomers bearing a side chain of medium chain length (mcl-PHAs) have been reported to exhibit low glass transition temperatures (T_g), and have been used as additives to reduce the stiffness of PLLA (Noda et al. 2004). Even when a small quantity (10–20 wt%) of mcl-PHA was mixed with PLLA, the properties of PLLA were dramatically improved. Evaluation of the miscibility of components in a polymer alloy is important for understanding the mechanism by which polymer properties are improved. Thus, a composite film composed of PLLA and PHA bearing fluorescein was prepared to determine the location of PHA. As expected, finely dispersed fluorescein was observed over the entire composite film by fluorescence microscopy (Fig. 7), thus confirming the suitability of this method for evaluating PHA dispersibility in a polymer alloy.

In conclusion, we have demonstrated the suitability of the UV-initiated thiol-ene click reaction for the functionalization of PHA. This is a simple and versatile methodology for the

preparation of functional PHAs, which was achieved via a three-step functionalization process, namely preparation of PHA containing vinyl groups, introduction of amino or carboxyl groups by the UV-initiated thiol-ene click reaction, and functionalization through subsequent chemical modification. A simpler and more versatile development of noble functional PHAs is now in progress in our group and will be reported in the near future. Our work therefore provides a rapid and facile method for evaluating the dispersibility of PHA in a polymer alloy without the requirement for special pre-treatment.

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Compliance with Ethical Standards

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure Captions

Fig. 1 Strategies for in vivo production of novel PHAs.

Fig. 2 ^{1}H NMR spectra of (a) PHAund60, (b) PHAund60-NH_2, (c) PHAund60-COOH, and (d) PHAund60-COOH with GRGDS (4). $CDCl_3$ and TMS were used as the solvent and internal standard, respectively. Units A and B shows 3HA and vinyl-bearing 3HA, respectively. C**H**(A3 and B3) means methyne protons of units A and B, and the numbers within the brackets mean the position of the carbon to which the protons bind. C$\mathbf{H_2}$(A2 and B2) means methylene protons of units A and B. C$\mathbf{H_2}$(B*) means methylene protons adjacent to the vinyl group.

Fig. 3 Introduction of thiol compounds into vinyl-bearing PHA via the thiol-ene click reaction.

Fig. 4 Modification of PHA with either fluorescein or a fibronectin active fragment (a GRGDS peptide).

Fig. 5 PHA yields and unit molar ratios of the vinyl group at various undecylenate concentrations.

Fig. 6 Fluorescence spectra of fluorescein-containing PHA (hollow circles) and free FITC (solid squares). Photographic images of (a) PHAund60-NH_2 and (b) Fluorescein-containing PHA under UV-irradiation.

Fig. 7 Photographic images of the fluorescent microscope observations of composite films, composed of (a) PLLA and PHAund60-NH_2 and (b) PLLA and PHA bearing fluorescein.

Fig. 8 Photographic images of light microscope observations of the 24-well plates coated with (a) PHAund60-COOH and (b) PHA containing GRGDS peptides after 6 h of cultivation.

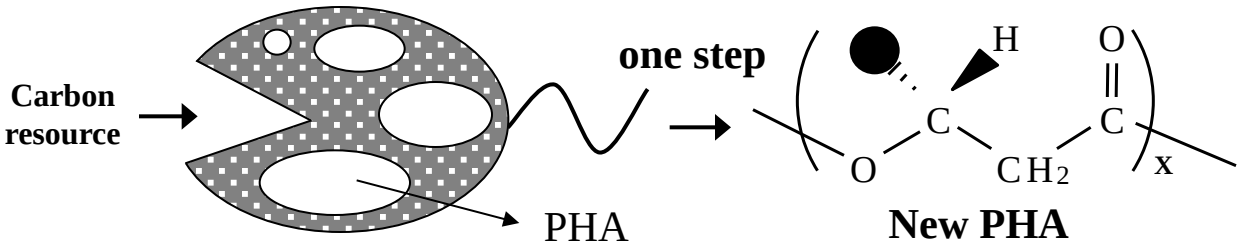
Table Titles

Table 1 Cell dry weights, PHA contents, and PHA yields for *P. putida* KT2440 grown under various undecylenate concentrations

Table 2 Monomer compositions and molecular weights of PHAs synthesized using *P. putida* KT2440 grown under various undecylenate concentrations

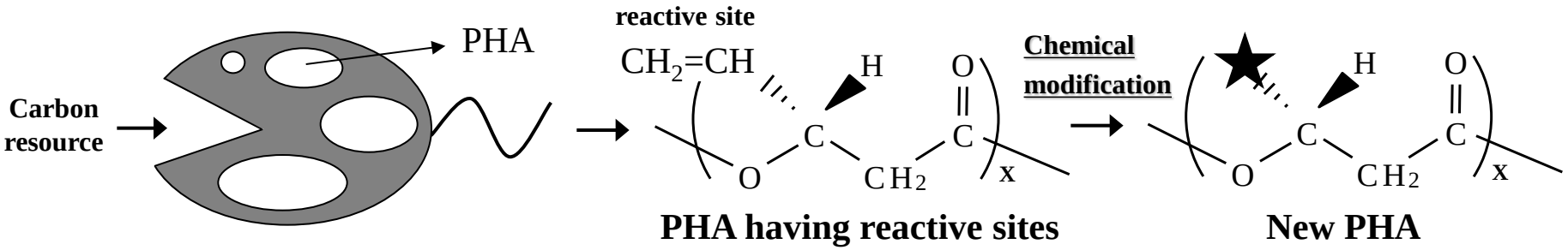
Fig. 1

Method 1: One-step synthesis of new PHAs



Advantage: One-step synthesis
Disadvantage: Search of a specific PHA synthase and construction of a supplying pathway for each new HA

Method 2: Multi-step synthesis of new PHAs



Advantage: Availability of synthesis of new PHAs with various structures and functions is possible.
Disadvantage: Multi-step synthesis

Fig. 2

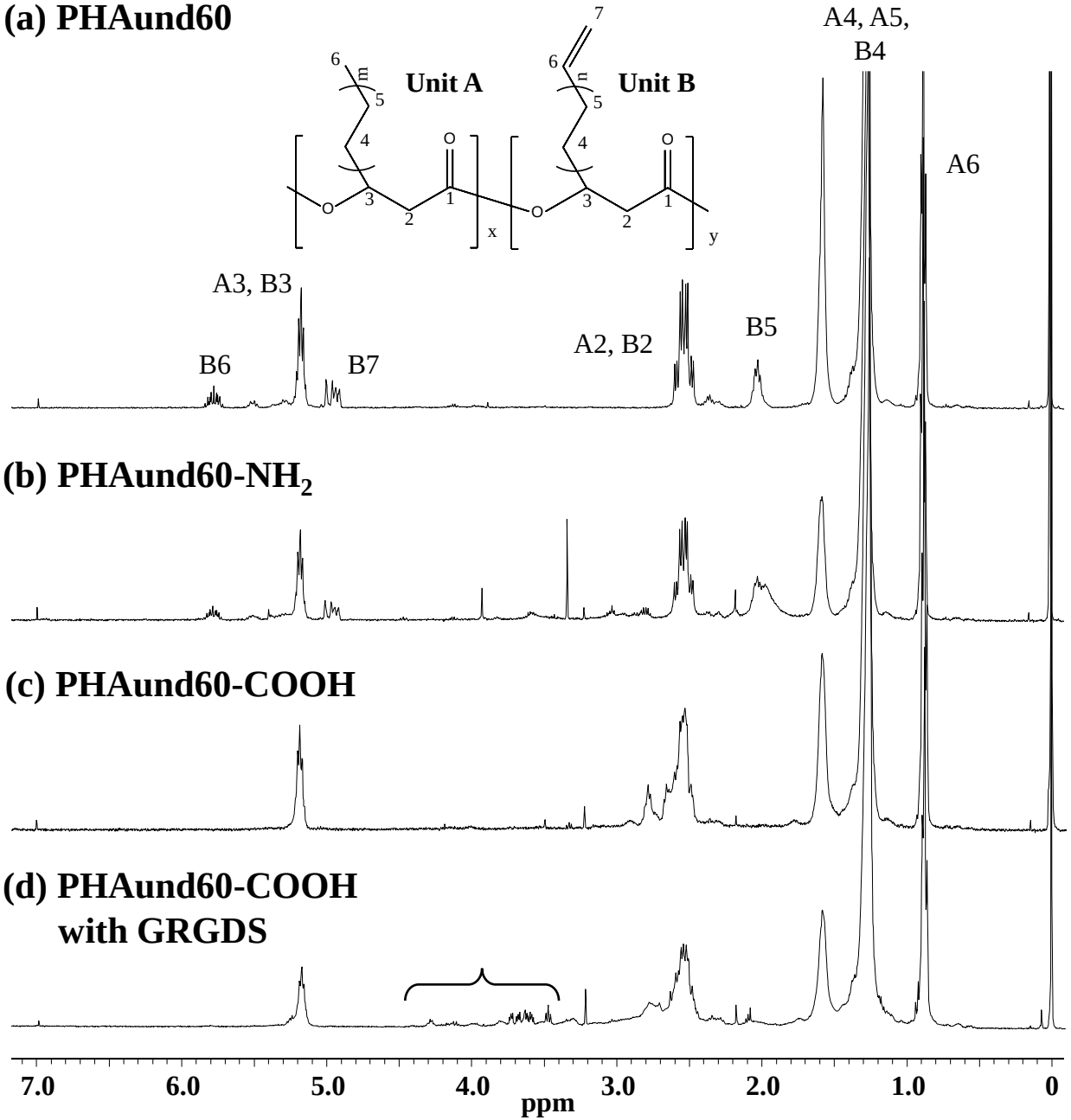
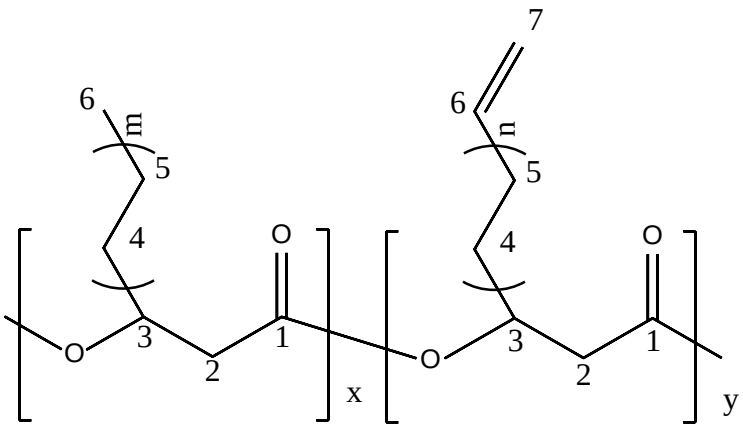

(a) PHAund60
Unit A
Unit B
A4, A5,
B4
A6
A3, B3
B6
B7
A2, B2
B5
(b) PHAund60-NH2
(c) PHAund60-COOH
(d) PHAund60-COOH
with GRGDS
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
ppm

Fig. 3

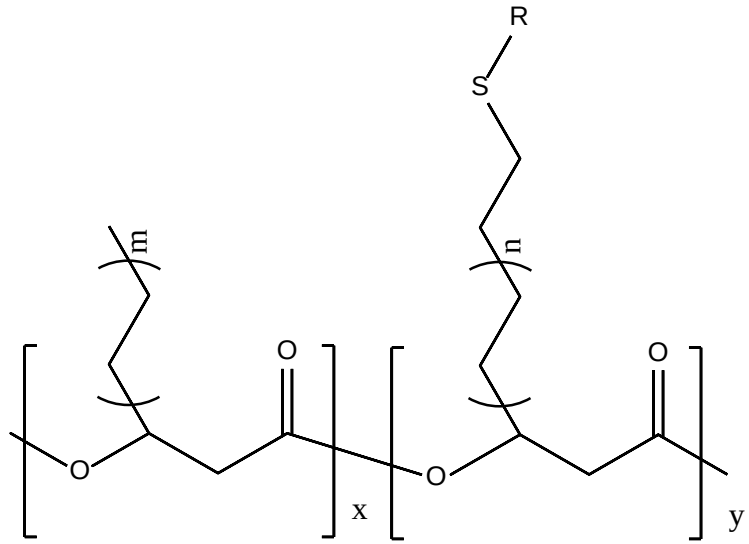


1
(m = 1 – 4, n = 1 – 3)

HS-R
$HSCH_2CH_2NH_2$
$HSCH_2CH_2COOH$

$h\nu$ (365 nm)
DMPA

Thiol-ene click reaction



2a R = $CH_2CH_2NH_2$
2b R = CH_2CH_2COOH
(m = 1 – 4 n = 1 – 3)

Fig. 4

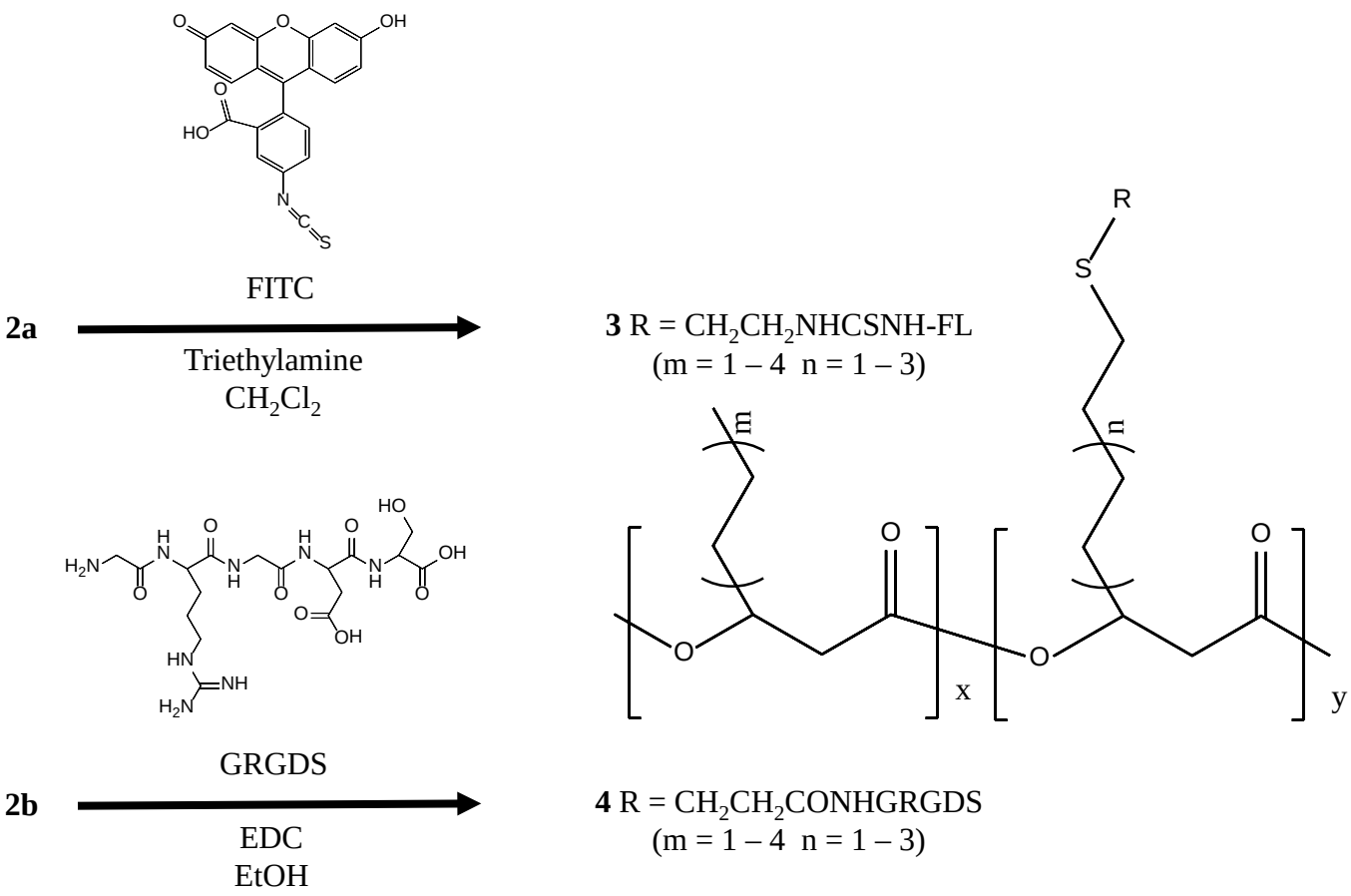

FITC
2a
Triethylamine
CH_2Cl_2
3 R = $CH_2CH_2NHCSNH$-FL
(m = 1 – 4 n = 1 – 3)
GRGDS
2b
EDC
EtOH
4 R = $CH_2CH_2CONHGRGDS$
(m = 1 – 4 n = 1 – 3)

Fig. 5

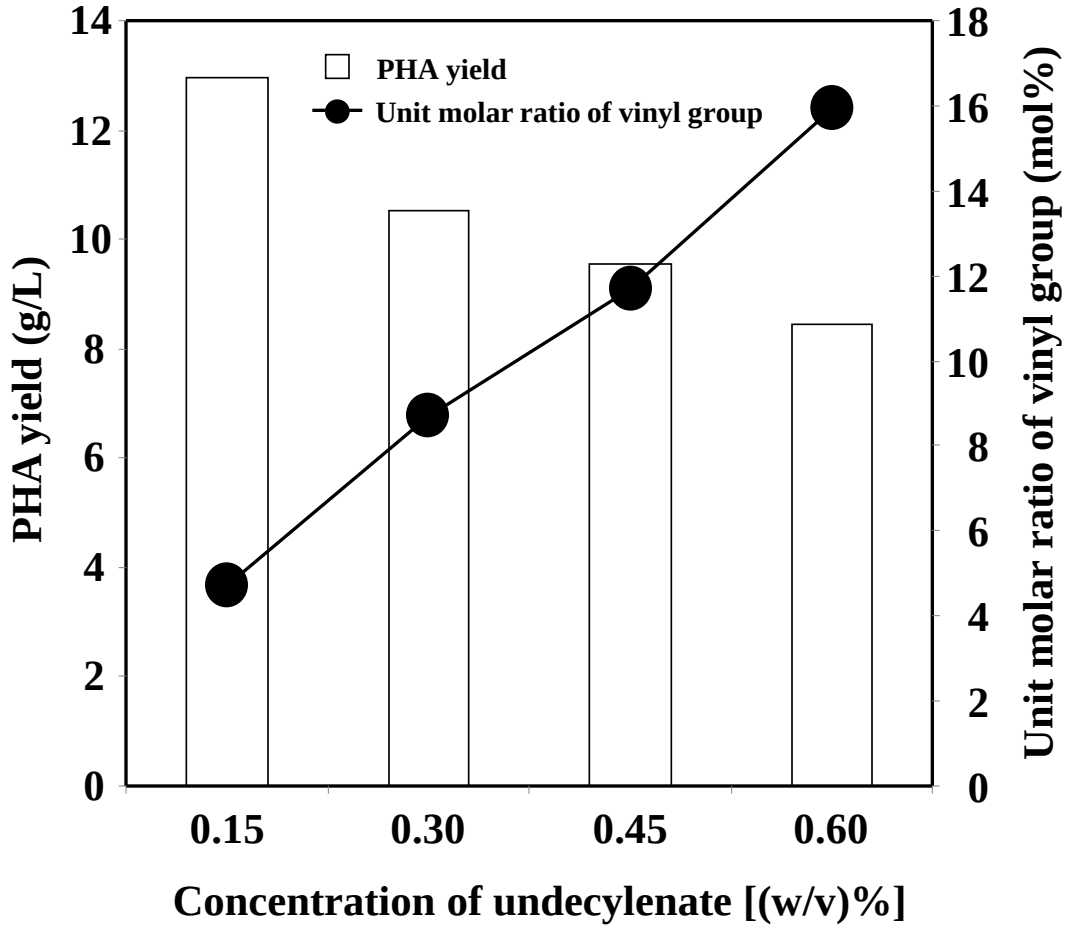

PHA yield
Unit molar ratio of vinyl group
PHA yield (g/L)
Unit molar ratio of vinyl group (mol%)
0
2
4
6
8
10
12
14
16
18
0.15
0.30
0.45
0.60
Concentration of undecylenate [(w/v)%]

Fig. 6

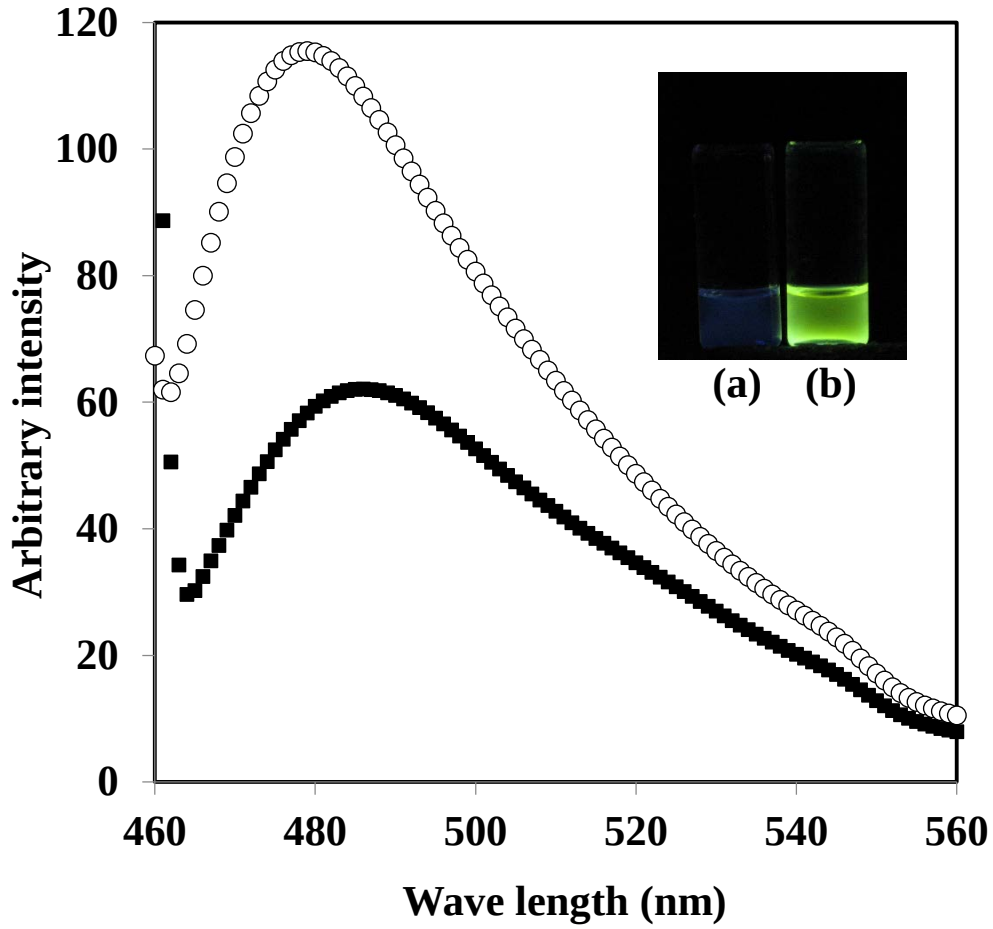

120
100
80
60
40
20
0
Arbitrary intensity
460
480
500
520
540
560
Wave length (nm)
(a)
(b)

Fig. 7

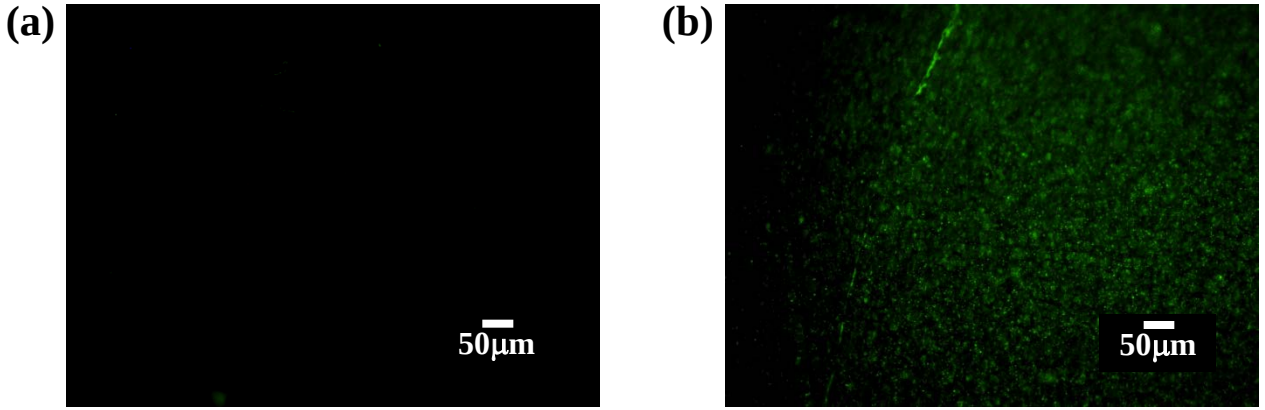


Fig. 8

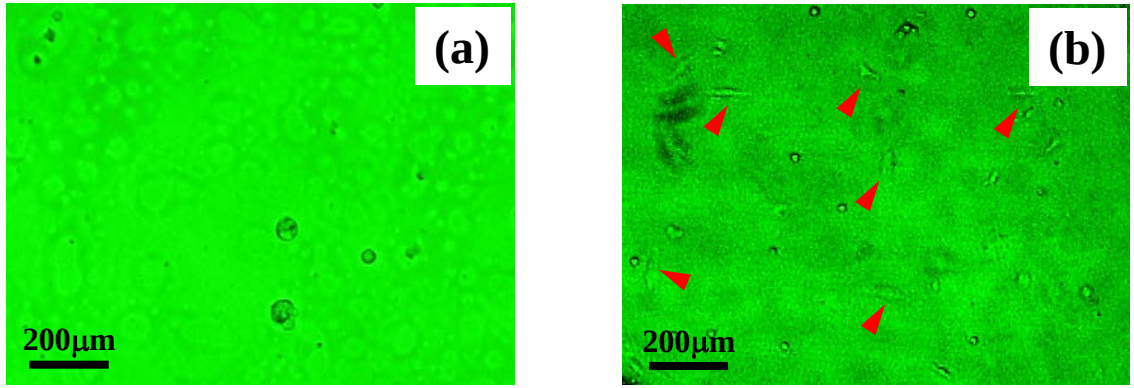


Table 1 Cell dry weights, PHA contents, and PHA yields for *P. putida* KT2440 grown under various undecylenoate concentrations.

Sample name	Concentration of undecylenoate %(w/v)	Cell dry weight g L^{-1}	PHA content[a] %	PHA yield g L^{-1}
PHAund15	0.15	40.5	32.0	12.9
PHAund30	0.30	32.5	32.4	10.5
PHAund45	0.45	27.5	34.7	9.5
PHAund60	0.60	30.2	28.0	8.5

[a]Determined by gas chromatography.

Table 2 Monomer compositions and molecular weights of PHAs synthesized by *P. putida* KT2440 grown under various undecylenoate concentrations.

Sample	Monomer composition[a] (mol%)									Molecular weight[b]		
name	3HB[d]	3HV	3HHx	C7:1	3HO	C9:1	3HD	C11:1	3HDD	Mw	Mn	*D*[c]
PHAund0	0	0	1.9	0	13.0	0	76.0	0	7.1	58,000	30,000	1.93
PHAund15	0	0	2.9	0	17.1	1.7	67.8	3.0	7.6	54,500	28,200	1.93
PHAund30	0	0	2.3	0	13.9	2.7	67.6	6.0	7.6	49,100	26,200	1.87
PHAund45	0	0	2.2	0	18.7	5.1	60.2	6.6	7.1	32,400	15,300	2.12
PHAund60	0	0	2.0	0.6	11.3	6.4	63.7	8.8	7.2	36,000	18,400	1.96

[a]Determined by gas chromatography.

[b,c]Determined by GPC using polystyrene samples as standards in chloroform.

[d]3HB = 3-hydroxybutyrate; 3HV = 3-hydroxyvaryrate; 3HHx = 3-hydroxyhexanoate; C7:1 = 3-hydroxy-6-heptenoate; 3HO = 3-hydroxyoctanoate; C9:1 = 3-hydroxy-8-nonenoate; 3HD = 3-hydroxydecanoate; C11:1 = 3-hydroxy-10-undecenoate; 3HDD = 3-hydroxydodecanoate.