Plastics Completely Synthesized by Bacteria: Polyhydroxyalkanoates

Guo-Qiang Chen

Contents

Abstract Polyhydroxyalkanoates (PHA) produced by many bacteria have been investigated by microbiologists, molecular biologists, biochemists, chemical engineers, chemists, polymer experts, and medical researchers over the past many years. Applications of PHA as bioplastics, fine chemicals, implant biomaterials, medicines, and biofuels have been developed. Companies have been established or involved in PHA-related R&D as well as large-scale production. PHA synthesis has been found to improve the robustness of non-PHA-producing microorganisms and to regulate bacterial metabolism, leading to yield improvement for some bacterial fermentation products. In addition, amphiphilic proteins related to PHA synthesis including PhaP, PhaZ, and PhaC have been found to be useful for achieving protein

G.-Q. Chen (✉)
Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing, 100084, China
e-mail: chengq@mail.tsinghua.edu.cn

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purification and even specific drug targeting. It has become clear that PHA and its related technologies are forming an industrial value chain ranging from fermentation, materials, and energy to medical fields.

1 Introduction

Polyhydroxyalkanoates (PHA), a family of biopolyesters with diverse structures, are the only bioplastics completely synthesized by microorganisms. PHA can be synthesized by over 30% of soil-inhabiting bacteria (Wu et al. 2000). Many bacteria in activated sludge, in high seas, and in extreme environments are also capable of making PHA. In the last 10 years, PHA have been developed rapidly to find applications in various fields (Fig. 1) (Chen 2009a).

PHA have rich properties depending on the structures (Figs. 2, 3). Homopolymers, random copolymers, and block copolymers of PHA can be produced depending on the bacterial species and growth conditions. With over 150 different PHA monomers being reported, PHA with flexible thermal and mechanical properties have been developed (He et al. 1999). Such diversity has allowed the development of various applications, including environmentally friendly biodegradable plastics for packaging purposes, fibers, biodegradable and biocompatible implants, and controlled drug

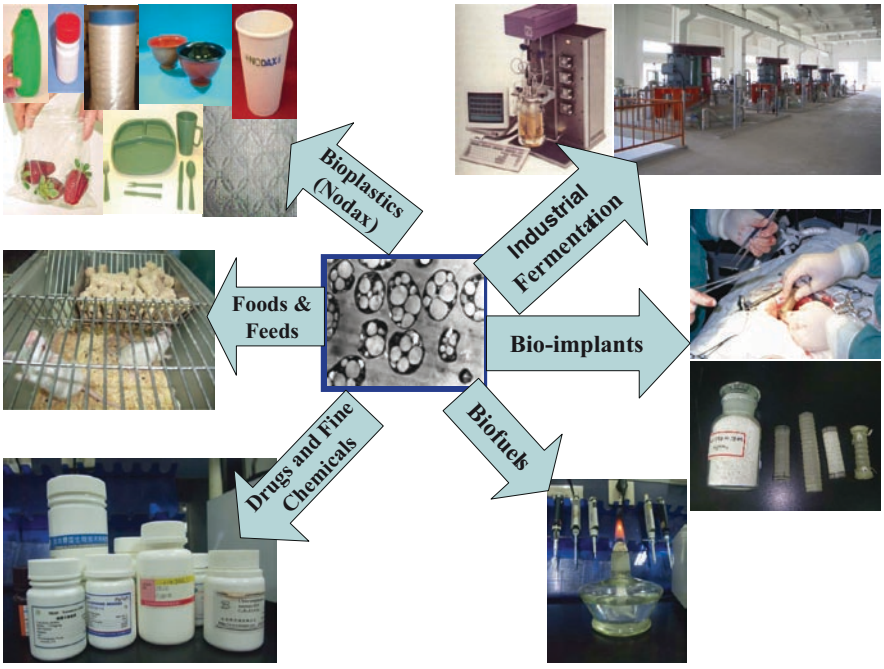

Fig. 1 Applications of polyhydroxyalkanoates (PHA) in various fields (Chen 2009a)

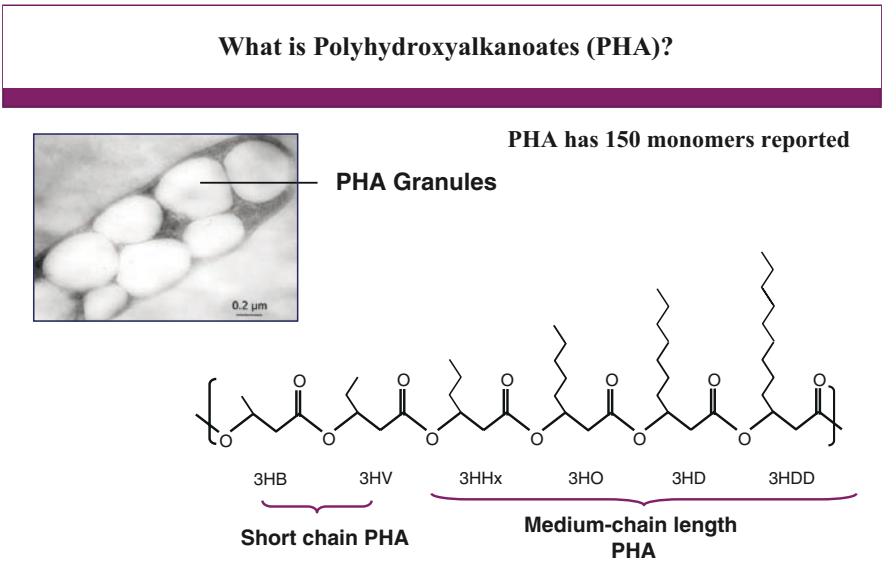

Fig. 2 Common PHA monomer structures. Short-chain-length monomers: 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV). Medium-chain-length monomers: 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDD)

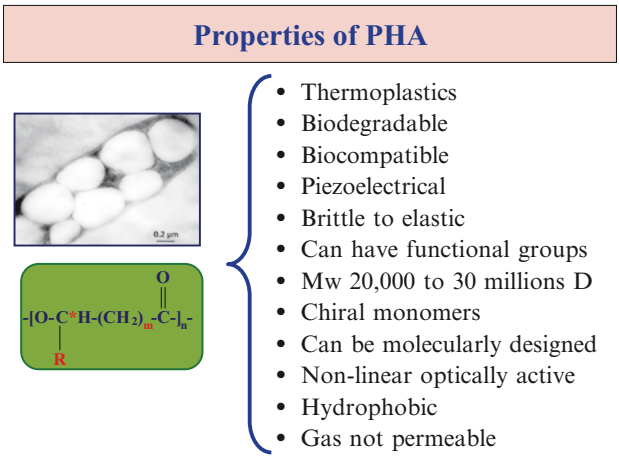

Fig. 3 Common properties of PHA

release carriers (Chen 2009a), PHA monomers can also be used to develop biofuels, drugs, or chiral intermediates. Oligomers of PHA were reported to be nutrients for animals (Tasaki et al. 1999).

Owing to these developments, microbial PHA has formed an industrial value chain ranging from industrial fermentation, materials, medicine, and biofuels to fine chemicals. More and more applications are the subject of intensive research. Globally, more than 20 companies have been established to commercialize these developments (Chen 2009a; Fig. 1).

In this chapter, we will discuss the above-mentioned aspects of PHA.

2 Biosynthesis of PHA

PHA can be synthesized either by chemical means or by biological approaches (Kemnitzer et al. 1993; He et al. 1999). Biosynthesis of PHA leads to much a higher molecular weight compared with that achieved with chemical methods. However, biosynthesis of PHA does not allow much control over the monomer structures in the PHA polymers; the specificity of PHA polymerase (or PHA synthase) will influence the monomers incorporated into the polymers (Chen et al. 2004). Since biosynthesis of PHA is conducted by microorganisms grown in an aqueous solution containing sustainable resources such as starch, glucose, sucrose, fatty acids, and even nutrients in waste water under 30–37 °C and atmosphere pressure, it is considered as more environmentally friendly and sustainable, especially when petroleum as a nonsustainable resource is being depleted quickly, and plastics or fuels based on petroleum show the same trend.

2.1 Biochemistry and Molecular Biology of PHA Synthesis

PHA biosynthesis has been well studied over the past many years. Acetyl-CoA is the key component to supply the 3-hydroxyalkanoyl-CoA of different lengths as substrates for PHA synthases of various specificities (Fig. 4, Table 1). In addition, 3-hydroxyalkanoyl-CoA can also be supplied from β-oxidation of fatty acids of different chain lengths (Fig. 4). Many genes encoding various enzymes are directly or indirectly involved in PHA synthesis (Table 1).

So far, biosynthesis of PHA can be summarized in eight pathways (Fig. 4, Table 1). The first pathway involves the three key enzymes β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHA synthase encoded by genes *phaA*, *phaB*, and *phaC*, respectively. *Ralstonia eutropha* is the representative of this pathway. An associated pathway involving PHA degradation catalyzed by PHA depolymerase, dimer hydrolase, 3-hydroxybutyrate dehydrogenase, and acetoacetyl-CoA synthase helps regulate PHA synthesis and degradation. The associated pathway was found in strains of *Aeromonas hydrophila*, *Pseudomonas stutzeri*, *R. eutropha*, and *Pseudomonas oleovorans* (Sudesh et al. 2000).

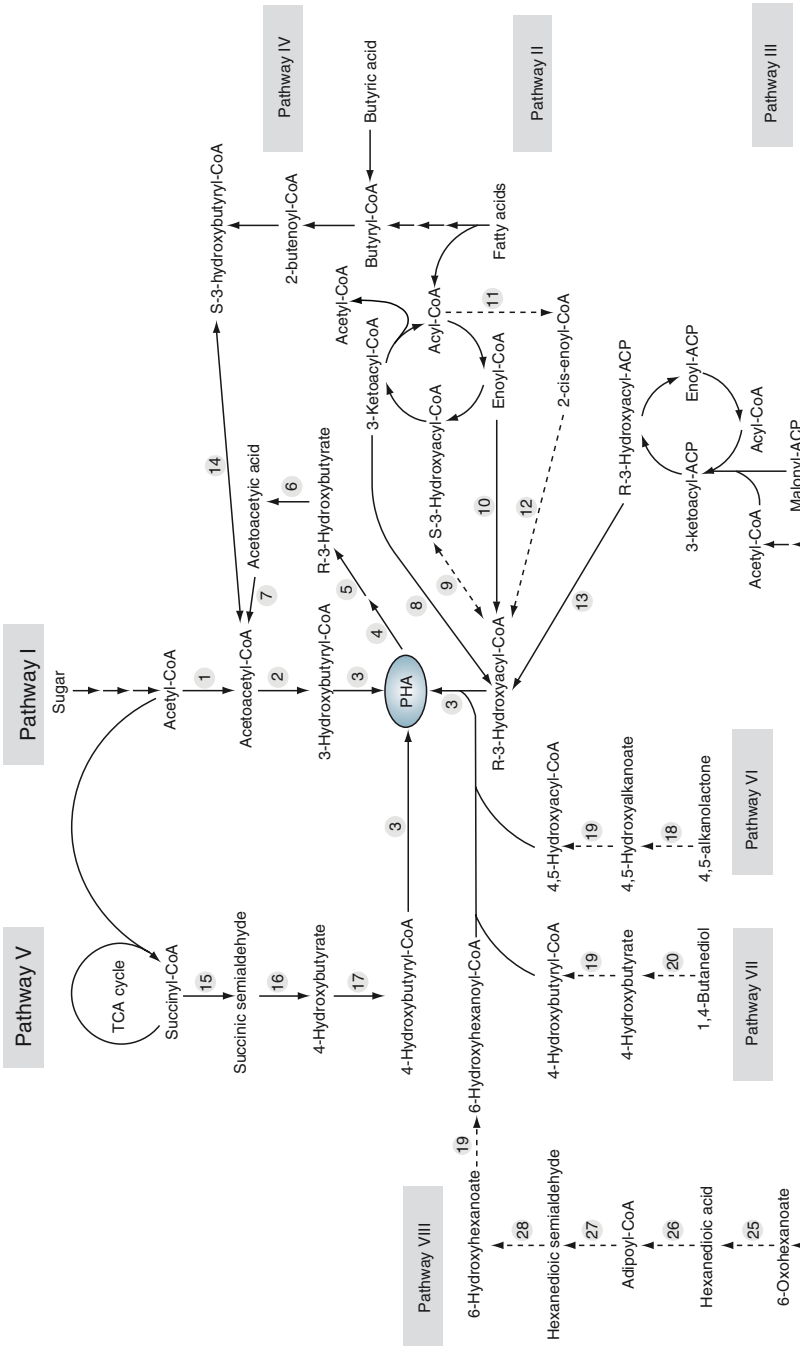

Fig. 4 PHA biosynthesis pathways. *Numbers* representing detailed enzymes (or genes) involved in the PHA synthesis can be obtained from Table 1

Table 1 Synthesis pathways for polyhydroxyalkanoates (*PHA*) and the enzymes involved

No.	Pathway	Abbreviation	Enzyme	Species	Reference
1	Pathway I	PhaA	β-Ketothiolase	*Ralstonia eutropha*	Sudesh et al. (2000)
2		PhaB	NADPH dependent acetoacetyl-CoA reductase		
3		PhaC	PHA synthase		
4	Associated way	PhaZ	PHA depolymerase	*Aeromonas hydrophila* 4AK4	Sudesh et al. (2000)
5			Dimer hydrolase	*Pseudomonas stutzeri* 1317	
6			(*R*)-3-Hydroxybutyrate dehydrogenase	*R. eutropha*	
7			Acetoacetyl-CoA synthetase	*Pesudomonas oleovorans*	
8	Pathway II	FabG	3-Ketoacyl-CoA reductase	*Pesudomonas putida* KT2442, *A. hydrophila* 4AK4, *Pesudomonas aeruginosa*	Sudesh et al. (2000), Mittendorf et al. (1998)
9			Epimerase		
10		PhaJ	(*R*)-Enoyl-CoA hydratase/enoyl-CoA hydratase I		
11			Acyl-CoA oxidase, putative		
12			Enoyl-CoA hydratase I, putative		
13	Pathway III	PhaG FabD	3-Hydroxyacyl-ACP-CoA transferaseMalonyl-CoA-ACP transacylase	*Pseudomonas mendocina*, recombinant *Escherichia coli*	Sudesh et al. (2000), Zheng et al. (2005), Taguchi et al. (1999)
14	Pathway IV		NADH-dependent acetoacetyl-CoA reductase	*Rhizobium* (Cicer) sp. CC 1192	Chohan and Copeland (1998)
15		SucD	Succinic semialdehyde dehydrogenase	*Clostridium kluyveri*	Valentin and Dennis (1997)
16	Pathway V	4hbD	4-Hydroxybutyrate dehydrogenase		
17		OrfZ	4-Hydroxybutyrate-CoA:CoA transferase		
18	Pathway VI		Lactonase, putative	Mutants and recombinant of *Alcaligenes eutrophus*	Valentin and Steinbüchel (1995)
19			Hydroxyacyl-CoA synthase, putative		
20	Pathway VII		Alcohol dehydrogenase, putative	*A. hydrophila 4AK4*	Xie and Chen (2008)
21	Pathway VIII	ChnA	Cyclohexanol dehydrogenase	*Acinetobacter* sp. SE19, *Brevibacterium epidermidis* HCU	Brzostowicz et al. (2002)
22		ChnB	Cyclohexanone monooxygenases		
23		ChnC	Caprolactone hydrolase		
24		ChnD	6-Hydroxyhexanoate dehydrogenase		
25		ChnE	6-Oxohexanoate dehydrogenase		
26			Semialdehyde dehydrogenase, putative		
27			6-Hydroxyhexanoate dehydrogenase, putative		
28			Hydroxyacyl-CoA synthase, putative		

The second PHA synthesis pathway (pathway II) is related to fatty acid uptake by microorganisms. After fatty acid β-oxidation, acyl-CoA enters the PHA monomer synthesis process. Enzymes including 3-ketoacyl-CoA reductase, epimerase, (*R*)-enoyl-CoA hydratase/enoyl-CoA hydratase I, acyl-CoA oxidase (putative), and enoyl-CoA hydratase I (putative) were found to be involved in supplying the PHA precursor 3-hydroxyacyl-CoA for PHA synthesis. *Pseudomonas putida*, *Pesudomonas aeruginosa*, and *A. hydrophila* are able to use pathway II to synthesize medium-chain-length (mcl) PHA or copolymers of (*R*)-3-hydroxybutyrate (R3HB) and (*R*)-3-hydroxyhexanoate (PHBHHx).

Pathway III involves 3-hydroxyacyl-ACP-CoA transferase (PhaG) and malonyl-CoA-ACP transacylase (FabD), which help supply 3-hydroxyacyl-ACP to form PHA monomer 3-hydroxyacyl-CoA, leading to PHA formation under the action of PHA synthase (Sudesh et al. 2000; Zheng et al. 2005; Taguchi et al. 1999).

Pathway IV uses NADH-dependent acetoacetyl-CoA reductase to oxidize (*S*)-(+)-3-hydroxybutyryl-CoA. A high ratio of NADPH to NADP+ could enhance the delivery of the reductant to nitrogenase in *Rhizobium* (Cicer) sp. strain CC 1192 (Chohan and Copeland 1998). This could also favor the reduction of acetoacetyl-CoA for poly[(*R*)-3-hydroxybutyrate] (PHB) synthesis.

Pathway V uses succinic semialdehyde dehydrogenase (SucD), 4-hydroxybutyrate dehydrogenase (4hbD), and 4-hydroxybutyrate-CoA:CoA transferase (OrfZ) to synthesize 4-hydroxybutyryl-CoA for forming 4-hydroxybutyrate-containing PHA. Pathway V was reported in *Clostridium kluyveri* (Valentin and Dennis 1997).

Pathway VI employs putative lactonase and hydroxyacyl-CoA synthase to turn 4,5-alkanolactone into 4,5-hydroxyacyl-CoA for PHA synthesis (Valentin and Steinbüchel 1995). Pathway VII is based on the putative alcohol dehydrogenase found in *A. hydrophila* 4AK4. In pathway VII, 1,4-butanediol is oxidized to 4-hydroxybutyrate, then to 4-hydroxybutyryl-CoA for 4-hydroxybutyrate-containing PHA synthesis (Xie and Chen 2008). Pathway VIII turns 6-hydroxy-hexanoate into 6-hydroxyhexanoate-containing PHA under the actions of eight enzymes (Table 1).

2.2 Prokaryotic PHA

Most PHA have been produced by prokaryotic microorganisms, including bacteria and archaea, although transgenic plants were reported to produce PHA (see Poirier and Brumbley 2009). Still, oligomers of PHA were reported to be discovered in eukaryotes, including many tissues and blood of human and animals (Reusch 1989). The functions of prokaryotic PHA were found to be related to carbon and energy storage as well as enhanced survival under environmental stress conditions (Castro-Sowinski et al. 2009). We humans exploit the fast growth of prokaryotes for our benefit to mass-produce PHA for applications as both bioplastics and biofuels. So far, all applications related to PHA are prokaryotic ones.

2.2.1 Homopolymer PHA

PHB was the first homopolymer PHA to be discovered. There have been very few studies related to other non-PHB homopolymers, including poly(4-hydroxybutyrate) (P4HB) (Steinbüchel et al. 1994), poly[(*R*)-3-hydroxyvalerate)] (PHV) (Steinbüchel and Schmack, 1995), poly[(*R*)-3-hydroxy-*co*-(*R*)-5-phenylvaleric acid] (Anderson et al. 1990), poly[(*R*)-3-hydroxyhexanoate] (Anderson et al. 1990), poly[(*R*)-3-hydroxyheptanoate] (Anderson et al. 1990; Chung et al. 1999; Wang and Chen 2009), poly[(*R*)-3-hydroxyoctanoate] (PHO) (Anderson et al. 1990), and poly[(*R*)-3-hydroxynonanoate] (Anderson et al. 1990; Chung et al. 1999). Many of these have not yet been fully characterized. Recently, the author's laboratory succeeded in producing poly[(*R*)-3-hydroxyundecanoate] and poly[(*R*)-3-hydroxydecanoate] (unpublished results). PHA homopolymers ranging from four to ten carbon atoms in length (or called C_4–C_{10} PHA homopolymers) have been produced. More homopolymers should be developed in the future.

Among these homopolymers, PHV can form solution-grown single crystals with a unique crystal and lamellar structure; this is very attractive for crystallography studies (Iwata and Doi 2000).

2.2.2 Copolymer PHA

In most cases, bacteria produce PHB. Also in many cases, short-chain-length (scl) PHA copolymers are synthesized consisting of C_3 and C_5, including poly[(*R*)-3-hydroxypropionate-*co*-(*R*)-3-hydroxybutyrate] (Shimamura et al. 1994), poly[(*R*)-3-hydroxybutyrate-*co*-4-hydroxybutyrate] (Saito et al. 1996), poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate] (PHBV) (Alderete et al. 1993), and poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate-*co*-4-hydroxybutyrate] (Zhao and Chen 2007). Many *Pseudomonas* spp. are able to accumulate mcl PHA copolymers containing C_6–C_{12} monomers. Typical mcl PHA are poly[(*R*)-3-hydroxyhexanoate-*co*-(*R*)-3-hydroxyoctanoate-*co*-(*R*)-3-hydroxydecanoate] and poly[(*R*)-3-hydroxyhexanoate-*co*-(*R*)-3-hydroxyl-octanoate-*co*-(*R*)-3-hydroxydecanoate-(*R*)-3-hydroxydodecanoate](Lageveen et al. 1988). Recently, the author's laboratory succeeded in producing poly[(*R*)-3-hydroxydecanoate-(*R*)-3-hydroxydodecanoate)] (unpublished results).

Copolymers of scl and mcl PHA possess useful and flexible mechanical properties; they are the preferred materials for application development. A successful example is the PHBHHx that was produced on an industrial scale (Chen 2009b). US-based Procter & Gamble has trademarked scl and mcl PHA copolymers of C_4 and C_6–C_{12} as Nodax™ (Noda et al. 2009).

2.2.3 Block Copolymer PHA

Pederson et al. (2006) synthesized PHA-containing block copolymers in *Cupriavidus necator* (also called *R. eutropha*) using periodic substrate addition. PHB segments

were formed during fructose utilization. Pulse feeds of pentanoic acid resulted in the synthesis of (*R*)-3-hydroxyvalerate (3HV) monomers, forming PHBV random copolymer. A combination of characterization techniques applied to the polymer batches strongly suggests the presence of block copolymers. Analysis of thermodynamically stable polymer samples obtained by fractionation by differential scanning calorimetry and nuclear magnetic resonance spectroscopy indicates that approximately 30% of the total polymer sample exhibits melting characteristics and nearest-neighbor statistics indicative of block copolymers. Rheology experiments indicate additional mesophase transitions only found in block copolymer materials. In addition, dynamic mechanical analysis shows extension of the rubbery plateaus in block copolymer samples, and uniaxial extension tests result in differences in mechanical properties (modulus and elongation at failure) expected of similarly prepared block copolymer and single polymer type materials.

McChalicher and Srienc (2007) showed that films consisting of block copolymers retained more elasticity over time with respect to films of similar random copolymers of comparable composition. Two PHBV films containing either 8 or 29% 3HV exhibited a quick transition to brittle behavior, decreasing to less than 20% elongation at fracture within a few days after annealing. Conversely, the block copolymer samples had higher than 100% elongation at fracture a full 3 months after annealing. Because block copolymers covalently link polymers that would otherwise form thermodynamically separate phases, the rates and degrees of crystallization of the block copolymers are less than those of the random copolymer samples. These differences translate into materials that extend the property space of biologically synthesized scl PHA.

Wu et al (2008) succeeded in producing PHB–poly(D,L-lactide) (PLA)–poly (ε-caprolactone) triblock copolymers using a low molecular weight methyl-PHB oligomer precursor as the macroinitiator through ring-opening polymerization with D,L-lactide and ε-caprolactone. The triblock copolymers exhibited flexible properties with good biocompatibility.

2.3 Eukaryotic PHA

PHB has been found to be a ubiquitous component of the cellular membranes of plants and animals (Reusch et al. 1992). The investigation of PHB distribution in human plasma using chemical and immunological methods found that PHB concentrations were highly variable: total plasma PHB ranged from 0.60 to 18.2 mg l^{-1}, with a mean of 3.5 mg l^{-1}, in a random group of 24 blood donors.

In plasma separated by density-gradient ultracentrifugation, lipoproteins constituted 20–30% of total plasma PHB, 6–14% was very low density lipoproteins (VLDL), 8–16% was low-density lipoproteins (LDL), and less than 3% was high density lipoproteins (HDL; Reusch et al. 1992). The majority of plasma PHB (70–80%) was found in protein fractions of density greater than 1.22 g ml^{-1}. Western blot analysis of the high-density fractions with anti-PHB F(ab')2 identified

albumin as the major PHB-binding protein. The affinity of albumin for PHB was confirmed by in vitro studies which demonstrated transfer of ^{14}C-PHB from chloroform into aqueous solutions of human and bovine serum albumins. PHB was less tightly bound to LDL than to other plasma components; the polymer could be isolated from LDL by extraction with chloroform, or by digestion with alkaline hypochlorite, but it could not similarly be recovered from VLDL or albumin. The wide concentration range of PHB in plasma, its presence in VLDL and LDL and its absence in HDL, coupled with its physical properties suggest it may have important physiological effects.

PHB of 130–170 monomer units is usually associated with other macromolecules by multiple coordinate bonds, or by hydrogen bonding and hydrophobic interactions (Reusch 1992). This conserved PHB has been isolated from the plasma membranes of bacteria, from a variety of plant tissues, and from the plasma membranes, mitochondria, and microsomes of animal cells.

PHB synthesis using genetic engineering approaches was reported in some plants, including switchgrass (Somleva et al. 2008), sugarcane (Purnell et al. 2007), sugar beet (Menzel et al. 2003), tobacco (Lossl et al. 2005), flax (Wrobel et al. 2004), *Arabidopsis thaliana* (Kourtz et al. 2005), rape, and corn (Poirier 2002).

3 Microbial Synthesis of PHA Monomers

Various enantiomerically pure (*R*)-3-hydroxyalkanoic acids (RHA) can be conveniently prepared by depolymerizing the biosynthesized PHA. De Roo et al. (2002) produced the chiral RHA and RHA methyl esters via hydrolytic degradation of PHA synthesized by pseudomonads. They first hydrolyzed the recovered PHA by acid methanolysis and then distilled the RHA methyl ester mixture into several fractions. Subsequently, the RHA methyl esters were saponified to yield the corresponding RHA with final yields of the RHA up to 92.8% (w/w).

3.1 PHA Monomers Produced by Microorganisms

Lee et al. (1999) demonstrated that R3HB could be efficiently produced via in vivo depolymerization by providing the appropriate environmental conditions. In their study with the strain *Alcaligenes latus*, they found that lowering the pH to 3–4 induced the highest activity of intracellular PHB depolymerase and blocked the reutilization of R3HB by the cells. Ren et al. (2005) suspended PHA-containing *P. putida* cells in phosphate buffer at different pH. When the pH was 11, the degradation and monomer release was the best. Under this condition, (*R*)-3-hydroxyoctanoic acid and (*R*)-3-hydroxyhexanoic acid were degraded with an efficiency of over 90% (w/w) in 9 h.

To produce extracellular chiral (*R*)-3-hydroxyacyl acids (3HA) by fermentation, a novel pathway was constructed by expressing *tesB* gene encoding thioesterase II into *P. putida* KTOY01, which was a PHA synthesis operon knockout mutant. A 0.35 $g l^{-1}$ 3HA mixture consisting of (*R*)-3-hydroxyhexanoate (3HHx), (*R*)-3-hydroxyoctanoate, (*R*)-3-hydroxydecanoate (3HD), and (*R*)-3-hydroxydodecanoate (3HDD) was produced in shake-flask study using dodecanoate as the sole carbon source. Additional knockout of *fadA* and *fadB* genes encoding (*R*)-3-ketoacyl-CoA thiolase and (*R*)-3-hydroxyacyl-CoA dehydrogenase in *P. putida* KTOY01 led to the weakening of the β-oxidation pathway. The *fadBA* and PHA synthesis operon knockout mutant *P. putida* KTOY07 expressing *tesB* gene produced 2.44 $g l^{-1}$ 3HA, significantly more than that of the β-oxidation intact mutant. The 3HA mixture contained 90% 3HDD as a dominant component. A fed-batch fermentation process carried out in a 6-l automatic fermentor produced 7.27 $g l^{-1}$ extracellular 3HA containing 96 mol% fraction of 3HDD after 28 h of growth. For the first time it became possible to produce 3HDD-dominant 3HA monomers (Chung et al. 2009).

3.2 The Application of PHA Monomers for Synthesis of Other Polyesters

Finally, the diverse PHA monomers are a rich pool for novel polymer synthesis (Taguchi et al. 2008; Rieth et al. 2002). Copolymerization of PHA monomers with commercially available polymer monomers will generate limitless new copolymers. This is an area that has not yet started to attract attention, possibly owing to the high cost of PHA monomer production. However, copolymer of lactide and 3-hydroxybutyrate (3HB) has recently been reported, signifying the start of the PHA monomer-based new polymer era.

4 Application of PHA

4.1 PHA as Packaging Materials

PHA were initially used to make everyday articles such as shampoo bottles and packaging materials by Wella (Germany) (Weiner 1997). PHA were also developed as packaging films mainly for uses as shopping bags, containers and paper coatings, disposable items such as razors, utensils, diapers, feminine hygiene products, cosmetic containers, and cups as well as medical surgical garments, upholstery, carpet, packaging, compostable bags and lids, or tubs for thermoformed articles by Proctor & Gamble, Biomers, Metabolix, and several other companies (Clarinval and Halleux 2005; Mikova and Chodak 2006).

PHB fibers with high tensile strength were prepared by stretching the fibers after isothermal crystallization near the glass-transition temperature (Tanaka et al. 2007).

Increasing the time for isothermal crystallization of PHB fibers resulted in a decrease in the maximum draw ratio. Yet the tensile strength of PHA fibers increased remarkably when the isothermal crystallization time was prolonged to more than 24 h. The tensile strength of low molecular weight drawn fibers was higher than that of high molecular weight fibers. PHB fibers stretched after isothermal crystallization had the oriented α-form crystal with the 2(1) helix conformation and the β-form with the planar zigzag conformation.

Vogel et al. (2007) attempted to use reactive extrusion with peroxide as a comfortable pathway for improvement of the crystallization of PHB in a melt spinning process. They succeeded in improving the crystallization in the spinline and of the inhibition of the secondary crystallization in the fibers. Those processes overcame the brittleness of PHA and created very strong fibers with promising applications.

4.2 PHA as Biomedical Implant Materials

Only several PHA, including PHB, PHBV, P4HB, PHBHHx, and PHO, are available in sufficient quantities for application research (Hrabak 1992; Byrom 1992; Chen et al. 2001). This is why most of the application research, including tissue engineering and controlled drug release, is based on the above-mentioned PHA.

Over the past 20 years, PHA and its composites have been used to develope devices including sutures, suture fasteners, meniscus repair devices, rivets, tacks, staples, screws (including interference screws), bone plates and bone plating systems, surgical mesh, repair patches, slings, cardiovascular patches, orthopedic pins (including bone filling augmentation material), adhesion barriers (Dai et al. 2009), stents, guided tissue repair/regeneration devices, articular cartilage repair devices (Wang et al. 2008a, b), nerve guides (Bian et al. 2009), tendon repair devices, atrial septal defect repair devices, pericardial patches, bulking and filling agents, vein valves, bone marrow scaffolds, meniscus regeneration devices, ligament and tendon grafts, ocular cell implants, spinal fusion cages, skin substitutes, dural substitutes, bone graft substitutes, bone dowels, wound dressings, and hemostats (Chen and Wu 2005). The changing PHA compositions also allow favorable mechanical properties, biocompatibility, and degradation times within desirable time frames under specific physiological conditions (Abe et al. 1995; Chen and Wu 2005).

In another study (Cheng et al. 2006a) it was shown that 3HB (0.02 g ml^{-1}) promoted cell proliferation in cultured L929 cells plated at high cell density (1×10^5 cells/well) but not at lower cell densities. Although 3HB did not affect cell cycle progression, it significantly inhibited cell death. 3HB treatment prevented necrosis, reducing cell membrane permeability 4 h following serum withdrawal from the medium, and for all subsequent time points. 3HB that promotes proliferation of L929 cells in high-density cultures by preventing apoptotic and necrotic cell death makes biodegradable polymers containing hydroxybutyrate, such as PHBHHx, attractive candidates for tissue engineering applications, especially those requiring the regeneration of large numbers of cells.

Cheng et al. (2006b) found that PHBHHx microparticles (0.005–0.10 g l^{-1}) promoted murine fibroblast L929 cell proliferation and elevated intracellular calcium concentrations. Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid inhibited PHBHHx-microparticle-induced cell proliferation by chelating the extracellular Ca^{2+} and blocking the PHBHHx particle-induced intracellular Ca^{2+} concentration increase. Transwell experiments demonstrated that PHBHHx microparticles stimulated fibroblast proliferation when separated from cells by a 0.4-μm filter as effectively as when applied directly to cells. Since the PHBHHx microparticles had a diameter of 75 μm, the stimulatory effect of PHBHHx particles on cell growth was attributed to degradation products smaller than 0.4 μm in diameter. The trophic effect of these microparticles is consistent with our previous reports demonstrating good biocompatibility for PHBHHx.

Oligo[(*R*)-3-hydroxybutyrate]s (OHBs; less than 14 kDa) existing in various organisms. They can form complexes with inorganic polyphosphates, nucleic acids, and proteins. OHBs are also the degradation products of PHB in vivo. Sun et al. (2007) prepared OHB (M_n 2,000), oligo[(*R*)-3-hydroxybutyrate-*co*-4-hydroxybutyrate] (O3HB4HB, M_n 2,100, 6 mol% 4-hydroxybutyrate), oligo[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate] (OHBHHx, M_n 2,800, 12 mol% 3HHx), and mcl oligo[(*R*)-3-hydroxyalkanoate]s (M_n 2,400, 71.2 mol% 3HD) via methanolysis of corresponding PHA polymers. The cells grew well in low-concentration (5 mg l^{-1}) liposomes containing the oligomers. Different cytotoxicity was exhibited after more oligomers (more than 20 mg l^{-1}) had been transported into the cells. The inhibition was decreased stepwise from OHB to OHD, as the monomer chain length increased. Compared with OHBHHx and OHD treatment, more cells arrested in G_0/G_1 phase, and died, probably induced by OHB and O3HB4HB. However, the cell death can be suppressed by R3HB released from the oligomers. It can be concluded that the more flexible chain combined with R3HB units had better biocompatibility and bioabsorbability. This can be a guide to select and develop new tissue engineering materials.

Besides, Ca^{2+} influx was also observed under a confocal laser scanning microscope in cells after transfection with oligomers–liposomes. It was presumed that not only OHB but also other OHA can form calcium channels in phospholipid bilayers, and can be incorporated into plasma membranes and had Ca^{2+} transport activity.

With successful approval of P4HB as an implant biomaterial by the FDA (http://www.tepha.com), more PHA-based biomaterials are expected to go into clinical trials soon. With the diversity of PHA materials, one can expect the PHA to become a family of bioimplant materials with rich applications.

4.3 PHA as Drug Delivery Carriers

Homopolymers and copolymers of lactate and glycolate are widely used in commercially available sustained release products for drug delivery. However, lactate and glycolate copolymers are degraded by bulk hydrolysis; hence, drug release cannot be

fully controlled (Pouton and Akhtar 1996). In the early 1990s, PHA became candidates for use as drug carriers owing to their biodegradability, biocompatibility, and degradation by surface erosion (Gould et al. 1987). PHA used as a drug carrier was reviewed in 1989 by Koosha et al (1989). The potential of matrices produced by direct compression of PHBV for oral administration has been proven with the benefits of simplified processing over alternative sustained release technologies (Gould et al. 1987). Increasing the polymer molecular mass caused an increased rate of sulfamethizole release from irregularly shaped PHB microparticles (Brophy and Deasy 1986). When the in vitro release and the in vivo release of the anticancer agent lomustine from PHB and PLA microspheres as potential carriers for drug targeting were compared, it was found that drug was released from the PHB microspheres faster (Bissery et al. 1985). Incorporation of ethyl esters or butyl esters of fatty acids into the PHB microspheres increased the rate of drug release (Kubota et al. 1988).

So far only PHB and PHBV have been studied for controlled drug release. It is expected that other PHA family members with diverse properties will bring more controlled release properties for the drug release field. This is still an area remaining to be exploited.

PHA granule binding protein PhaP is able to bind to hydrophobic polymers (Wang et al. 2008b). A receptor-mediated drug-specific delivery system was developed in this study based on PhaP (Fig. 5). The system consists of PHA nanoparticles, PhaP, and ligands fused to PhaP. The PHA nanoparticles were used to package mostly hydrophobic drugs, PhaP fused with ligands produced by overexpression of their corresponding genes in *Pichia pastoris* or *Escherichia coli* was able to attach to hydrophobic PHA nanoparticle. At the end, the ligands were able to pull the PhaP–PHA nanoparticles to the targeted cells with receptors recognized by the ligands. It was found in this study that the receptor-mediated drug-specific delivery

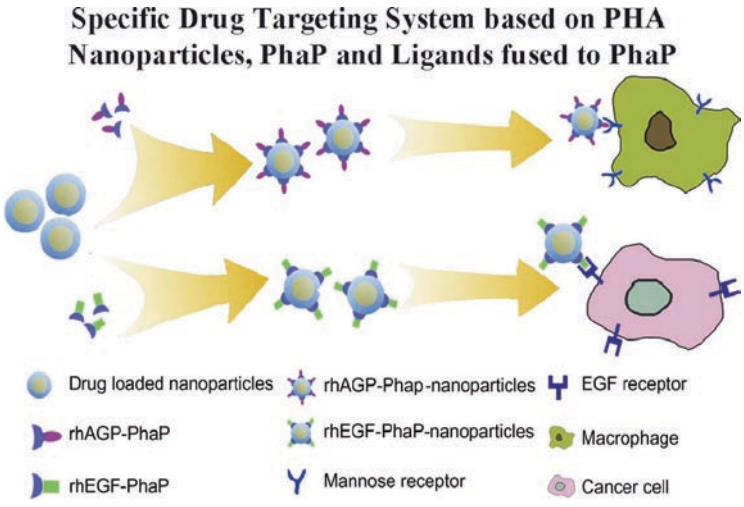

Fig. 5 PHA- and phasing-based specific drug delivery systems (Wang et al. 2008b)

system ligand–PhaP–PHA nanoparticles was taken up by macrophages, hepatocellular carcinoma cell BEL7402 in vitro, and hepatocellular carcinoma cells in vivo, respectively, when the ligands were mannosylated human α_1-acid glycoprotein and human epidermal growth factor (hEGF), respectively, which were able to bind to receptors of macrophages or hepatocellular carcinoma cells. The system was clearly visible in the targeted cells and organs under fluorescence microscopy when rhodamine B isothiocyanate (RBITC) was used as a delivery model drug owing to the specific targeting effect created by specific ligand and receptor binding. The delivery system of hEGF–PhaP–nanoparticles carrying RBITC was found to be endocytosed by the tumor cells in an xenograft tumorous model mouse. Thus, the ligand–PhaP–PHA specific drug delivery system was proven effective both in vitro and in vivo (Yao et al. 2008).

4.4 PHA as Biofuels

Recently, Zhang et al (2009) showed that 3-hydroxybutyrate methyl ester (3HBME) and mcl 3-hydroxyalkanoate methyl ester (3HAME) obtained from esterification of PHB and mcl PHA could be used as biofuels (Fig. 6). They investigated the combustion heats of 3HBME, 3HAME, ethanol, *n*-propanol, *n*-butanol, 0# diesel,

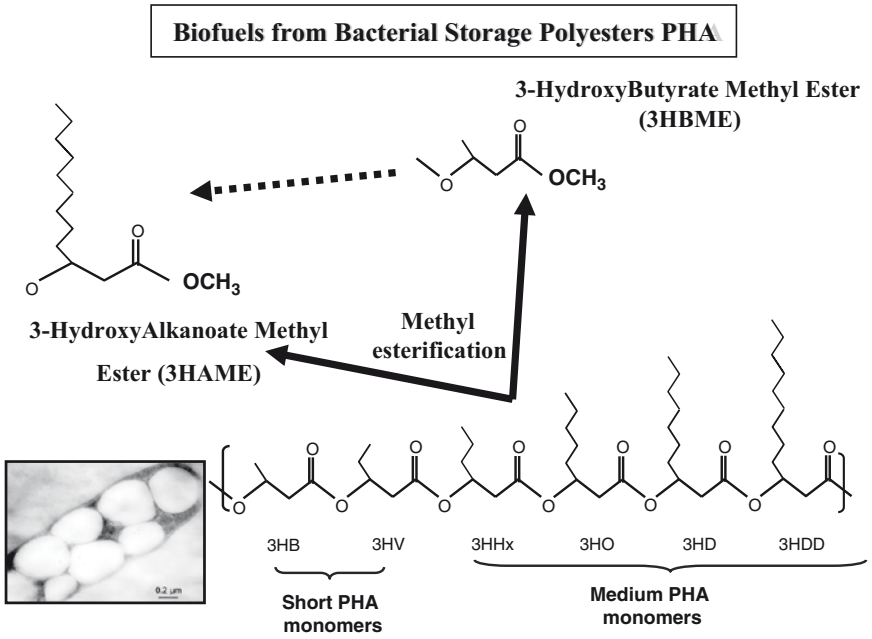

Fig. 6 PHA-based biofuels derived from methyl esterification of various PHA monomers (Zhang et al. 2009)

90# gasoline, and 3HBME-based and 3HAME-based blended fuels and found that 3HBME and 3HAME had combustion heats of 20 and 30 kJ g^{-1}, respectively, comparable to the combustion heat of 27 kJ g^{-1} of ethanol. Addition of 10% 3HBME or 3HAME enhanced the combustion heat of ethanol to 30 and 35 kJ g^{-1}, respectively. The addition of 3HBME or 3HAME to *n*-propanol and *n*-butanol led to a slight reduction of their combustion heats. The combustion heats of the blended fuels 3HBME/diesel or 3HBME/gasoline and of 3HAME/diesel or 3HAME/gasoline were lower than that of the pure diesel or gasoline. It was roughly estimated that the production cost of PHA-based biofuels should be around US $1,200/ton.

4.5 PHA Monomers as Drugs

Sodium salts of D-3-hydroxybutyrate (D-3HB), DL-3-hydroxybutyrate (DL-3HB), and 3HBME are derivatives of 3HB, a body ketone that is produced in vivo in animals, including human. D-3HB is the most common degradation product of microbial PHA that have been investigated for tissue engineering applications. 3HB and its derivatives (collectively called 3HB derivatives) were reported to have an effect on cell apoptosis and the cytosolic Ca^{2+} concentration of mouse glial cells (Xiao et al. 2007). The percentage of cells undergoing apoptosis decreased significantly in the presence of 3HB and its derivatives, as evidenced by flow cytometry. The in vitro study on the cytosolic Ca^{2+} concentration demonstrated that 3HB derivatives elevated dramatically the cytosolic Ca^{2+} concentration. Both the extracellular and the intracellular Ca^{2+} contributed as sources of such Ca^{2+} concentration elevation. The effect of 3HB derivatives on cytosolic Ca^{2+} concentration could be reduced by nitredipine, an L-type voltage-dependent calcium channel antagonist. In comparison, 3HBME worked more efficiently than D-3HB and DL-3HB did as 3HBME is most efficient in permeation into the cells. All the results indicated that 3HB derivatives had an inhibitory effect on cell apoptosis which is mediated by signaling pathways related to the elevation of cytosolic Ca^{2+} concentration. This positive effect helps explain the biocompatibility observed for PHA; it also points to the possibility of 3HB derivatives regardless of chirality becoming effective neural protective agents.

Learning and memory require energy-demanding cellular processes and can be enhanced when the brain is supplemented with metabolic substrates. It was found that neuroglial cell metabolic activity was significantly elevated when neuroglial cells were cultured in the presence of the PHB degradation product 3HB and derivatives. We demonstrated that the receptor for 3HB, namely, protein upregulated in macrophages by interferon-γ (PUMA-G), was expressed in brain and upregulated in mice treated with 3HBME. We also affirmed increased expression of connexin 36 protein and phosphorylated extracellular-signal-regulated kinase 2 (ERK2) in brain tissues following 3HBME treatment, although these differences were not statistically significant. Mice treated with 3HBME performed significantly ($p < 0.05$) better in the Morris water maze than either the negative controls (no treatment) or the positive controls (acetyl-L-carnitine treatment). Moreover, 3HBME was observed to

enhance gap junctional intercellular communication between neurons. Thus, 3HB and its derivatives enhance learning and memory, possibly through a signaling pathway requiring PUMA-G that increases protein synthesis and gap junctional intercellular communication (Zou et al. 2009).

5 Conclusion and Future Perspectives

The development of PHA into a branch of bulk chemical industry will address at least three issues: shortage of petroleum for plastic materials, reduction of CO_2 emissions, and environmental protection. It is related to the sustainable development of the chemical and material industries. The newly developed PHA-based biofuels open up a new area for development that avoids argument on food versus fuel and fuel versus land. However, much more work needs to be done to reduce the cost of PHA production so that PHA-based biofuel can be added to the existing bio-based fuels, including ethanol, propanol, butanol, biodiesel, hydrogen, and methane gas (Fig. 7).

High-value-added PHA applications should be developed simultaneously, especially the implant biomaterials that have begun to be recognized by the FDA. In addition, chiral monomers should be further exploited for medical usages (Fig. 7). So far, only 3HB and its derivatives have been studied and have revealed obvious therapeutic efforts, more monomers should be tested for medical efficacy.

The PHA surface binding proteins, including PhaP, PhaZ, and PhaC, can be developed into a protein purification system or specific drug delivery tools. More applications based on these proteins should be developed.

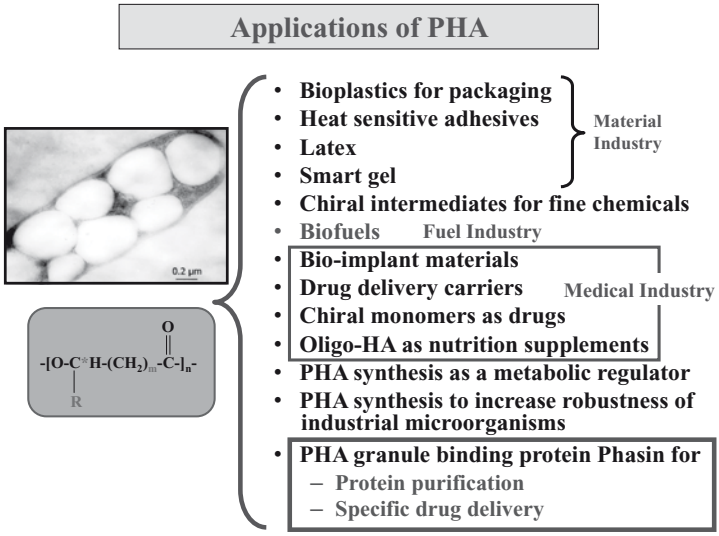

Fig. 7 PHA has been developed into an industrial value chain

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