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Controlled Synthesis of Polyhydroxyalkanoic (PHA) Nanostructures in *R. eutropha*

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ABSTRACT

In biological systems the detailed structure of macromolecules is usually encoded genetically. Here we show that the synthesis and structure of certain biopolymers can be directed by taking control of the reaction pathways in microorganisms to direct the reaction kinetics. The biological synthesis of polyhydroxyalkanoates (PHAs) presents a unique opportunity to deposit synthesized polymer into controlled nanostructures with features as small as the molecular length scale. Furthermore, this control can be extended to the level of individual polymer molecules, enabling the synthesis of block copolymers that could be used in combination with self-assembly methods to generate specific structures.

Background. PHAs are biodegradable polyesters synthesized intracellularly by many different bacterial species. A growth limitation by a nutrient other than the carbon source causes polymer synthesis as a store of carbon and energy.[1] PHA polymerization in *Ralstonia eutropha* is catalyzed by a synthase enzyme that resides on the surface of the polymer granule and adds monomer units to the growing polymer chain. The synthase enzyme is able to add different monomer units depending upon which external carbon sources are available to the bacterium.

Introduction. Limited examples of PHA microstructure synthesis have been previously reported. Core and shell granules were synthesized in *Pseudomonas oleovorans* by Curley et al. (1996).[2] Horowitz and Sanders (1994) were also able to show phase separation in reconstituted PHA granules.[3] However, none of these methods allowed for temporal control of alternating polymer synthesis or for multiple changes in synthesis conditions. Previous attempts to control the molecular structure of PHAs to obtain block copolymers was not successful, and it was concluded that in vivo block copolymer synthesis was not possible. The detailed view of PHA synthesis kinetics has been controversial, further clouding the possibility of block copolymer synthesis. Doi and others presented experimental data supporting the idea that PHA synthesis involves a rapid turnover of the biopolymers.[4] The cellular accumulation of the polymer would result from a shift of the balance between synthesis and degradation toward synthesis. However, others have shown that this mechanism may not be correct.[5] Recently, an in vitro enzymatic technique has been developed and used to synthesize PHA block copolymers.[6] This technique is likely to advance the study and use of PHA block copolymers. However, it suffers from the very high cost of the chiral monomers and purified enzyme used for in vitro synthesis. The presented work builds upon our first reporting of successful synthesis of core and shell granules in *R. eutropha*.[7]

Results. The approach described in this paper allows for control of polymer microstructures down to the nanometer length scale. The nature of the extracellular carbon source available to *R. eutropha* has a direct effect on which intracellular monomers are available to be incorporated into the growing polymer chain. For instance, in this work, extracellular fructose is metabolized by the bacteria to form intracellular hydroxybutyrate monomers. These monomers are used to synthesize homopolyhydroxybutyrate (PHB). However, an odd-chained fatty acid, such as valeric acid, is required for *R. eutropha* to synthesize hydroxyvalerate monomers. Switching metabolism back and forth between (i) excess fructose with limiting valeric acid and (ii) only fructose presents results in the synthesis of polyhydroxybutyrate-*co*-valerate (PHBV) and PHB homopolymer, respectively (Figure 1A). The resultant phase-separated nanostructures are dependent on the number of valerate additions. A single addition results in the synthesis of core and shell granules. Multiple additions lead to multilayered granules, as detailed below.

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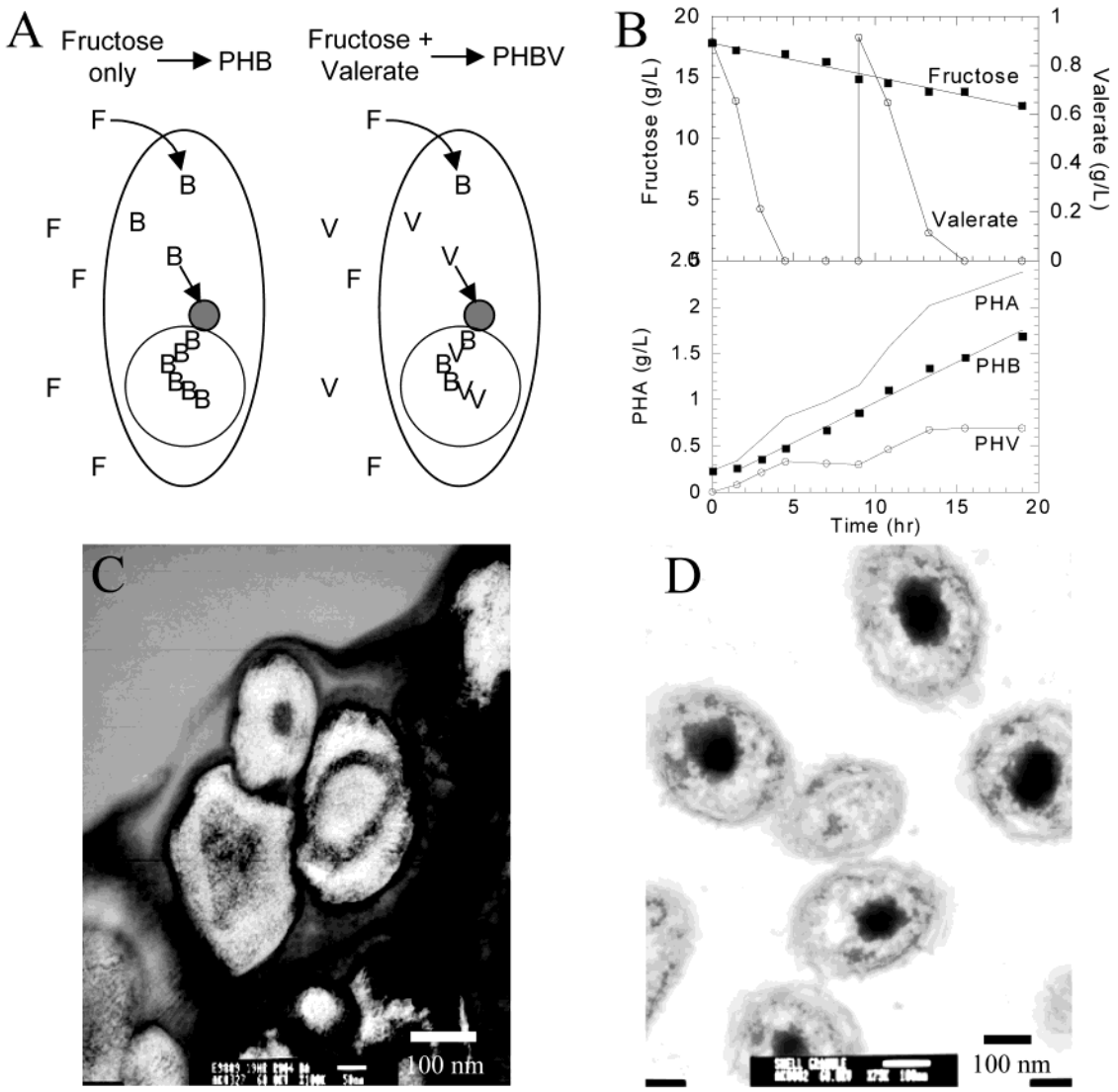

Figure 1. Synthesis of controlled microstructures in *R. eutropha*. (A) The two different media conditions lead to the synthesis of different polymers. Fructose is used to synthesize PHB homopolymer while the addition of valeric acid produces a random PHBV copolymer. (B) Bioreactor data for synthesis of multilayered granules. Valerate (1 g/L) was added to the bioreactor at 0 and 9 h, while fructose was present in excess. The PHA synthesized appears in discrete intervals as PHBV, PHB, PHBV, and PHB, respectively. (C) TEM imaging of multilayered granules after RuO_4 staining. The PHBV copolymer stains black with the PHB homopolymer appearing a lighter gray. (D) Core and shell granules similarly stained with Pb-citrate, uranyl-acetate, and OsO_4.

Figure 1B shows bioreactor data from the synthesis of multilayered granules. With valerate additions at 0 and 9 h, the external environment goes through four synthesis regimes, two each for the synthesis of copolymer PHBV and homopolymer PHB. The hydroxyvalerate monomers are synthesized only while valeric acid is present in the media. The synthesis of multilayered granules was confirmed by transmission electron microscopy (TEM, Figure 1C and 1D). The polymer granules were stained with RuO_4 to develop contrast between the different polymer phases.[8−10] PHBV appears darker than the PHB, presumably because its lower crystallinity allows a greater penetration of the heavy metal stain.

The control of granule microstructures can be directly extended to individual polymer chains. If switches in the available carbon source are made at least once during the synthesis of each chain, different blocks of the chain will contain different polymer types. Because it was unclear whether a detectable amount of block copolymer could be synthesized biologically, a mathematical model of the system was constructed to simulate the synthesis kinetics (manuscript submitted). The model results in the following population balance describing the dynamics of the molecular weight distribution of polymer chains that change in time t according to

$$\frac{\partial A(x,t)}{\partial t} + \frac{\partial}{\partial x}[R_{el}(x,S)A(x,t)] + R_t(x,S)A(x,t) = 0$$

$$\frac{\partial I(x,t)}{\partial t} = R_t(x,S)I(x,t)$$

where $A(x,t)$ and $I(x,t)$ are the molecular weight distributions of active and inactive chains, respectively. R_{el} is the elongation rate and R_t the termination rate. In the general

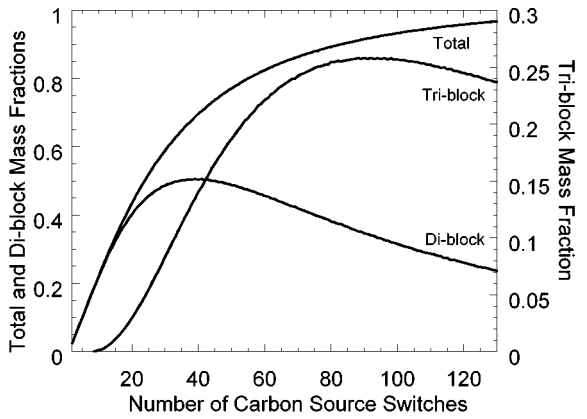

Figure 2. Modeling of block copolymer synthesis in *R. eutropha*. There is an optimum number of switches to maximize a certain type of block copolymer chain, such as diblock copolymers. The model predicts that 34 switches would produce 50% diblock copolymer of the total synthesized polymer.

case the rates depend on the molecular weight x of a chain and on the monomer concentration S. The initiation rate R_i contributes to the model through the boundary condition.

This model was used to optimize the number of switches to maximize the production of diblock and triblock copolymer chains (Figure 2).[11] Using the conditions optimized for diblock copolymer synthesis, experimental confirmation was attempted with *R. eutropha* using valerate additions every 55 min (Figure 3A). The uptake and exhaustion of valeric acid could be efficiently followed by monitoring the dissolved oxygen concentration (DO) within the bioreactor, because cells consume more oxygen when both the fatty acid and fructose are utilized concomitantly. To determine how much block copolymer was synthesized, the recovered polymer was fractionated into homo-PHB, block copolymer, and PHBV random copolymer fractions (Figure 3B). Even after repeated fractionations, it was not possible to separate the block copolymer fraction into its PHB and PHBV component parts, in contrast to blends made as controls (data not shown). Both PHB and PHBV melting peaks are shown in the differential scanning calorimetery (DSC) trace in Figure 3C, confirming that the covalent bond between the two phases of the block copolymer prevent fractional separation of the components. Nuclear magnetic resonance predicted the composition of the block copolymer fraction as 56% PHB and 44% PHBV copolymer (data not shown). Finally, the block copolymer sample was annealed for five minutes at 200 °C, stained with RuO_4, and examined with a TEM showing co-continuous phase separation (Figure 3D). After examination of successive fractionations it was determined that 7−14% of the synthesized polymer was indeed block copolymer.

Discussion. It is increasingly desirable to miniaturize materials and devices in an effort to get more work done or store more information in a smaller space. The technique of metabolic switching has been shown to control microstructures on the order of 25 nanometers for multilayered granules and potentially down even smaller for block copolymers. These structures could find uses as scaffolding for nanotechnology. Extension of this technique to include many of the more than 90 different monomers that have been detected in PHAs would allow for many different active substituents on the polymer chain.[12] These could be used to attach and grow other microstructures, depending upon the need. Multilayered spheres have also shown promise for controlled drug release, something for which PHAs have already been considered.[13,14] The ability to control the microstructure of PHAs at such a small length scale shows many exciting opportunities for this material in the near future.

Experimental. *Bioreactor. R. eutropha* H-16 was cultivated on complex media in increasing volumes by inoculating with 1% volume from previous cultures.[15] Ultimately the cells were added to a 5 L tower reactor (New Brunswick Scientific Co., Inc., Edison, NJ) with complex media and 4 g/L $(NH_4)_2SO_4$ and allowed to grow to an optical density of 27 at 433 nm (9−12 h), corresponding to late exponential growth. Three liters of cells were collected, centrifuged at 4000 rpm (Beckman JA-10 rotor 2800 g) for 10 min, and washed twice with sterile phosphate buffer (0.036 M, pH 7.0). Polymer synthesis occurred in a 5 L B. Braun Biotech Inc. (Allentown, PA) bioreactor with 3 L of minimal media with just enough nitrogen (0.02 g/L $(NH_4)_2SO_4$) to allow the cells to adapt to new carbon source (Friedrich et al., 1981). Agitation was maintained at 600 rpm at 30 °C. H_2SO_4 (1.8 vol %) was used to maintain the pH at 7.0. The reactor started with 20 g/L fructose which remained present in excess throughout. Valeric acid was added intermittently as outlined below.

Valerate Additions. The MCFS control system (B. Braun Biotech Inc., Allentown, PA**)** was programmed to add 20 mL of 15.04 g/L sodium valerate solution at an interval supplied by the operator. For this work, valerate was added every 55 min.

Gas Chromatography (GC) Analysis of PHAs. All polymer samples were analyzed by propanolysis of the monomers and detected by GC according to standard protocols.[16]

Polymer Isolation from Biomass. Cells were harvested at the conclusion of the fermentation, decanted, and washed with three volumes of acetone. After centrifugation (4000 g for 10 min), the acetone was removed and the biomass was allowed to dry for several days in a fume hood. The dried biomass was broken up and placed in a cellulose thimble (25 mm × 80 mm, Whatman, Springfield Mill in Maidstone Kent, UK). The thimble was used in a Soxhlett apparatus with hot chloroform to extract the soluble polymer from the residual biomass overnight. PHA was precipitated by adding four volumes of methanol to the chloroform extract and recovered by filtration.

Polymer Fractionation. Polymer blends were fractionated into their component parts by dissolving the polymer in chloroform at 5 g/L. Heptane was slowly added until a precipitate formed.[17] This process was continued until polymer no longer precipitated from the solution.

Differential Scanning Calorimetry (DSC). Melting points were measured on a liquid nitrogen cooled DSC (Pyris model, Perkin-Elmer, Norwalk, CT). Approximately 10 mg of polymer was sealed in an aluminum pan (Perkin-Elmer)

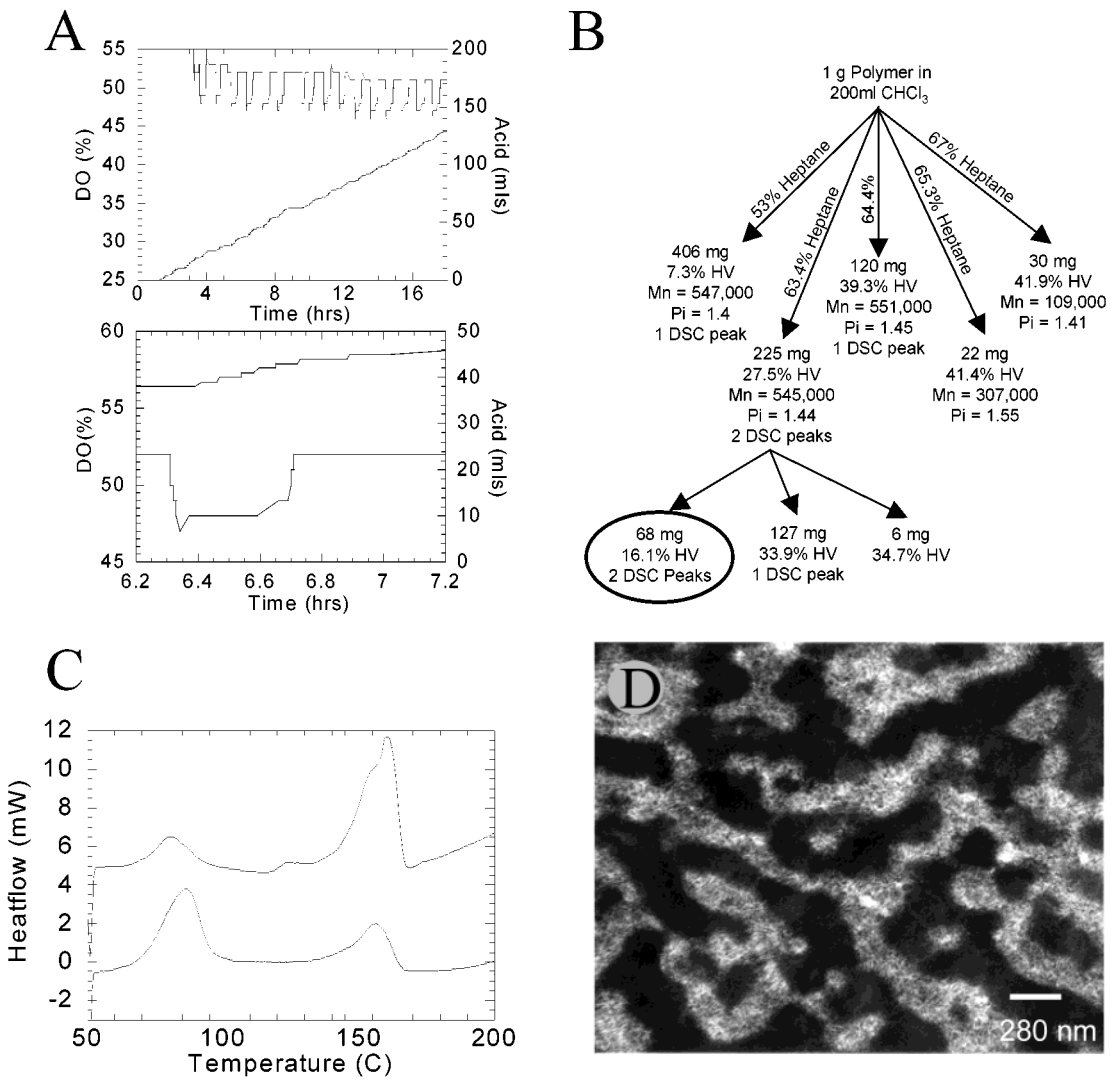

Figure 3. In vivo synthesis of block copolymers. (A) Valeric acid was added every 55 min (dashed lines). Dissolved oxygen (DO) decreased upon valerate addition due to the increase in oxygen demand by the culture (lower curve). (B) Fractionation of the isolated polymer. Increasing concentrations of heptane were required to precipitate chains with higher valerate (HV) monomer concentration. Molecular weight did not affect the fractionation until no difference in HV% remained (fractions 4 and 5). The second fraction showed two melting peaks during DSC, indicating it contained some block copolymer. Further fractionation of that sample yielded some random copolymer mixed with the block copolymer. Mn = number average molecular weight, Pi = polydispersity index. (C) DSC scans of polymer fraction 2 in B. The upper trace is after the first fractionation showing two melting peaks, one at 85 °C and the second at 160 °C, supporting the presence of block copolymers in the sample. The lower trace is the sample circled in B, and after a second fractionation both melting peaks are still present in the sample, confirming the presence of block copolymers. (D) TEM micrograph of the isolated block copolymer stained with RuO_4.

and melted at 200 °C for one minute. The sample was then allowed to cool to 80 °C and held for three or more days to expedite crystallization. Samples were heated at 20 °C/min. from 50 °C to 200 °C to measure melting points.

Nuclear Magnetic Resonance (NMR). Polymer samples were dissolved in $CDCl_3$ (Sigma) at 40−50 mg/ml. C^{13} signals were detected on a Varian Inova 125 MHz NMR. Monomer sequence distributions were used to detect random copolymers or blends of copolymers using methods outlined by Kamiya et al.[18]

Transmission Electron Microscopy (TEM). Samples (30−50 mg) were sealed in 1 mL glass vials that had been flushed with argon. The vials were placed in an oil bath at 200 °C for 5 min then quenched in liquid nitrogen. Small pieces of polymer were fixed in Quetol 651 embedding epoxy by incubating at 60 °C overnight (Ted Pella, Redding, CA). The samples were stained with RuO_4 as outlined previously.[7]

Molecular Weight Determination. Molecular weight distributions of PHAs were determined by gel permeation chromatography. Samples were dissolved at 2−3 mg/mL chloroform and analyzed on a Waters 150C GPC system equipped with a refractive index detector and two columns: a Phenomenex Phenogel 5×10^5 angstrom column and a Perkin-Elmer PLGel 10 μ column, connected in series. Injection volumes of 100 μL were used with chloroform as the eluent at 1 mL/min. Polystyrene standards were used to create a universal calibration curve and correlated to PHA with the proper Mark−Houwink parameters.[19−20]

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