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Letter

Extracellular Poly(hydroxybutyrate) Bioplastic Production Using Surface Display Techniques

Kevin Beaver, Ashwini Dantanarayana, Willisa Liou, Markus Babst, and Shelley D. Minteer*

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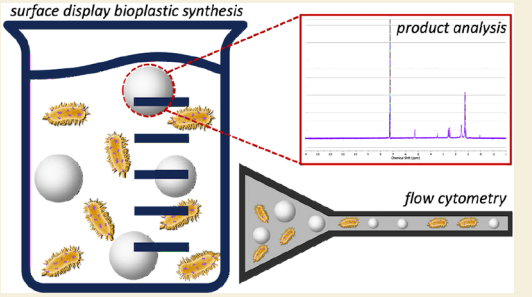
ABSTRACT: Poly(hydroxybutyrate) is a biocompatible, biodegradable polyester synthesized naturally in a variety of microbial species. A greener alternative to petroleum-based plastics and sought after for biomedical applications, poly(hydroxybutyrate) has failed to break through as a leading material in the plastic industry due to its high cost of production. Specifically, the extraction of this material from within bacterial cells requires lysis of cells, which takes time, uses harsh chemicals, and starts the process again with growing new living cells. Recently, surface display of enzymes on bacterial membranes has become an emerging technique for extracellular biocatalysis. In this work, a fusion protein lpp-ompA-phaC was expressed in *Escherichia coli* to display the enzyme poly(hydroxyalkanoate) synthase on the cell surface. The resulting poly(hydroxybutyrate) product was chemically characterized by nuclear magnetic resonance and infrared spectroscopy. Finally, the extracellular synthesis of the bioplastic granules was demonstrated qualitatively via microscopy and quantitatively by flow cytometry. The results of this work are the first demonstration of extracellular synthesis of poly(hydroxybutyrate), showing promise for continuous and scalable synthesis of materials using surface display.



KEYWORDS: *surface display, biopolymer synthesis, poly(hydroxybutyrate), NMR, FT-IR, flow cytometry, microscopy*

INTRODUCTION

Over billions of years, microorganisms have evolved biosynthetic methods for polymer materials as a form of energy storage and resistance to osmotic stress.[1] Among these bioplastics, poly(hydroxybutyrate) (PHB) has been commercially produced since the 1980s, and PHB has emerged as an optimal poly(hydroxyalkanoate) material for a wide range of applications.[2] This is due to its unique combination of advantageous properties, including water-insolubility, high melting point, biocompatibility, and biodegradability.[3,4] With these properties, PHB and its copolymers are sought after for biomedical applications like absorbable sutures and screws, in addition to being greener alternatives to petroleum-based plastics for packaging, adhesives, and fabrics.[5]

Originally prevalent in the microbial fermentation industry, PHB production is most commonly performed in a number of bacterial species, notably in *Cupriavidus necator* (formerly referred to in the literature as *Alcaligenes eutrophus*, *Watersia eutropha* and *Ralstonia eutropha*),[6] *Bacillus* spp.,[7] and cyanobacteria.[8] Bacteria are effective PHB producers, synthesizing up to 80% PHB in their dry biomass,[9] but PHB production is limited by the small cell size and requires cell lysis to obtain the product. Specifically, it has been estimated up to 50% of the polymer's relatively high cost is due to the expensive processes required to disrupt and separate the bacterial cells from the PHB granules.[10]

In efforts to move away from bacteria, their PHB biosynthetic pathways have been engineered into eukaryotic hosts like diatoms[11] and plants,[12] but these systems have drawbacks in lower relative yield and larger space requirements. Specifically, there is high interest in bioinspired methods, like using poly(hydroxyalkanoate) synthase (phaC) to polymerize hydroxybutyryl coenzyme A monomers into PHB.[13] Further, an *in vitro* synthetic approach utilized a purified enzyme cascade including phaC to produce PHB in cell-free synthesis, but methods like this one tend to have shortcomings due to enzymes having brief lifespans and high purification costs.[3] In order to bypass these issues, hybrid strategies can be envisioned that use living organisms to express phaC and produce PHB extracellularly in order to address the cell lysis problem and create a simple, continuous biosynthetic pathway.

Cellular surface display of enzymes is a growing field that uses host organisms to express peptides extracellularly, typically for use in vaccine development, epitope screening, and bioremediation.[14] More recently, biocatalysis applications

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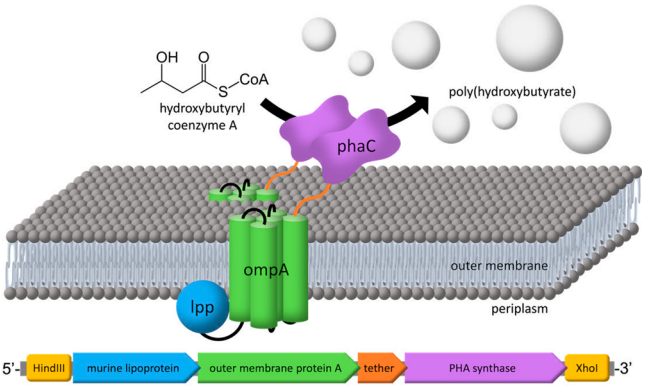
for surface display have been demonstrated, for example, in microbial fermentation[15] and fuel cell chemistry.[16] Compared to enzymatic secretion, surface display offers many advantages including enhanced stability in varying temperature and pH, as well as increasing protein−protein interactions and substrate transfer.[17] Surface display synthesis of materials is not yet well-studied, but the potential of enzymatic surface display systems in biocatalysis opens opportunities for extracellular synthesis of complex molecules like polymers.

Scheme 1. Fusion Protein Sequence Based on lpp-ompA Surface Display Strategy with Flexible Tether[a]



[a]PHA synthase (phaC) is expressed for extracellular synthesis of poly(hydroxybutyrate).

While there are many unique strategies to achieve surface display, one that has gained much attention for displaying large proteins (up to 74 kDa) in Gram-negative hosts is a fusion to lipoprotein−outer membrane protein A (lpp-ompA).[14] This method uses a lipoprotein anchor to the periplasmic side of the bacterial outer membrane and fuses to a portion of *Escherichia coli* (*E. coli*) outer membrane protein A with 5 transmembrane regions.[18] This traditional method has been built upon by the addition of a C-terminal flexible tether region to allow the passenger protein to be displayed further from the membrane (up to 90 Å), reducing steric hindrance.[19] Since phaC is known to dimerize in nature,[6] this flexible tether could allow the surface displayed enzymes conformational flexibility to dimerize (Scheme 1). In addition, the catalytic domain of the phaC enzyme is the C-terminal region,[20] so this surface display strategy allows the phaC protein to be fused by its N-terminus to the C-terminus of the flexible tether.

In this work, *E. coli* is used as a model host for surface display of phaC via lpp-ompA. By addition of the PHB precursor hydroxybutyryl CoA (HbCoA), synthesis of PHB is confirmed via chemical characterization methods, including nuclear magnetic resonance and infrared spectroscopy. Finally, extracellular synthesis of PHB is suggested by various microscopy methods and flow cytometry. To the best of our knowledge, this is the first demonstration of extracellular bioplastic material synthesis, using a surface display approach.

RESULTS AND DISCUSSION

First, the polymerized PHB product of engineered *E. coli* lpp-ompA-phaC was characterized according to its chemical functional groups. By far the most popular characterization method in the literature, ^{1}H NMR was used to characterize the PHB standard (Figure S1), the HbCoA standard, and the biosynthesized products of *E. coli* lpp-ompA-phaC and *E. coli* wild-type that were provided the PHB precursor HbCoA. When solvated in $CDCl_3$, PHB shows a unique signal at 5.25 ppm, a sextuplet corresponding to the lone hydrogen on the asymmetric carbon. The hydrogens of the CH_2 group also show a unique shift at 2.45−2.65 ppm, as a doublet of quadruplets. Finally, perhaps the most difficult peak to discern is the doublet at 1.26−1.28 ppm, corresponding to the hydrogens of the methyl group.

When compared to the PHB standard, the product of *E. coli* lpp-ompA-phaC produced the sextuplet at 5.25 ppm and the doublet of quadruplets from 2.45−2.65 ppm, suggesting that the product was indeed PHB (Figure 1). It also appears that the triplet peak at 5.35 ppm, which can be seen clearly in the HbCoA standard, as well as a slightly visible shift in the *E. coli* wild-type confirms that some leftover HbCoA precursor, or other coenzyme A products were contaminating the *E. coli* lpp-ompA-phaC sample. The methyl peaks for PHB are seen in *E. coli* lpp-ompA-phaC at 1.28 ppm, but the other peak at 1.26 ppm is strongly seen in all the samples (likely due to other methyl-containing contaminants, Figure S2), making this shift not unique to PHB. Altogether, the ^{1}H NMR data strongly suggest that PHB was synthesized by the *E. coli* lpp-ompA-phaC, but not in the wild-type.

Solid samples of the PHB standard, *E. coli* PHB+ pellet (containing both the bacteria and biosynthesized PHB), and *E. coli* PHB− pellet (containing only bacteria, since HbCoA was

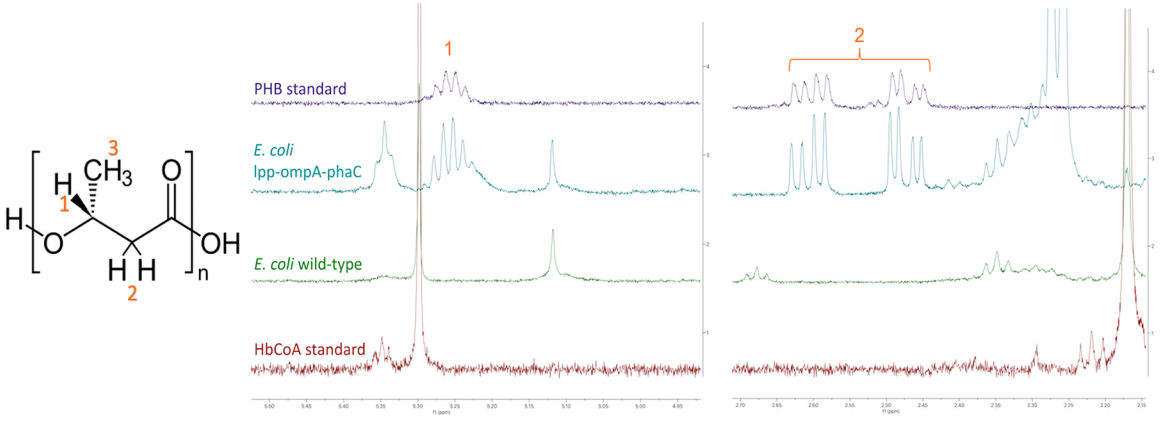


Figure 1. NMR analysis was performed for PHB and HbCoA standards, as well as products obtained from the engineered and wild-type *E. coli* cells.

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not provided) were examined on the FT-IR ATR (Figure 2). In the PHB standard, the most resolved peak at 1720 cm^{-1}

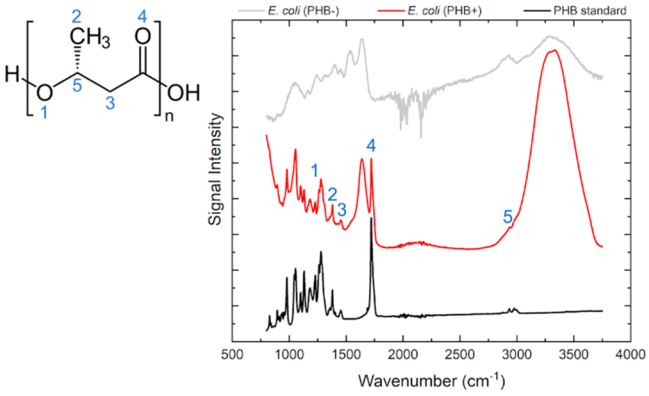

Figure 2. FT-IR analysis of PHB standard, as well as pellets obtained from centrifuging PHB+ and PHB− treatments of *E. coli* lpp-ompA-phaC.

corresponds to the carbonyl functional group of the ester linkage in the polymer. The peak at 1230 cm^{-1} corresponding to the C−O bond of the ester is observed to a lesser degree. The CH_3 peak is seen at 1380 cm^{-1} and the CH_2 peak is observed at 1450 cm^{-1}. Finally, the 2930 cm^{-1} peak corresponds to the CH of the asymmetric carbon. While a few of these peaks are seen in all 3 samples, even in the *E. coli* PHB− sample, the most distinguishable signal at 1720 cm^{-1} and the peak at 1380 cm^{-1}, representing the carbonyl and methyl functional groups, respectively, appear only in the PHB standard and the *E. coli* PHB+ sample. The *E. coli*-related peaks, including the strong signal at 1645 cm^{-1} and the broad peak at 3300 cm^{-1}, are seen only in the bacterial samples and not the PHB standard. Together, these results suggest that the pellet of *E. coli* PHB+ contained both the PHB polymer and bacterial cells.

After chemically characterizing the polymer produced by *E. coli* lpp-ompA-phaC, it was vital to confirm that the polymer was being produced extracellularly. In addition to visible observation of a bright-white pellet in the *E. coli* PHB+ sample, microscopy and flow cytometry were used to confirm extracellular synthesis of PHB. Three different microscopy methods were used, with unique advantages and disadvantages. For instance, scanning electron microscopy (SEM) of PHB standard and *E. coli* PHB+ suspensions dried on a silicon wafer allowed surface characterization of extracellular PHB granules (Figure S3). However, this method did not allow the bacteria to retain their true shape, as can be seen in some other sample preparation methods. Transmission electron microscopy (TEM) using aldehyde-fixed and osmium tetroxide treated cells allowed better resolution of cell structure and visualization of extracellular polymer formation (Figure S4). Fluorescence microscopy allowed live cell imaging using Nile red stain to weakly stain bacterial membranes and strongly stain PHB granules (Figure S5). However, the bright-field fluorescence microscope did not allow a high enough resolution for reliable size analysis of either the cells or PHB granules.

After qualitative analysis by microscopy, flow cytometry was performed for quantitative analysis of the cell cultures. Flow cytometry has been used to study intracellular PHB production, such as in *C. necator* and *S. meliloti*.[21] In flow cytometry, forward light scattering (FSC) is related to cell size, while side light scattering (SSC) is related to cell granularity or opacity. In addition, fluorescence filters can be used to determine the degree of staining. As shown in the microscopy, and from previous literature,[21] Nile red stains PHB granules more strongly than bacterial cells. The flow cytometry method was first tested on the known intracellular PHB producer *C. necator*. As expected, the FSC was not significantly different for PHB+ and PHB− *C. necator* cells, since the cells retain normal size as they store PHB intracellularly (Figure S6). However, a statistically significant increase in fluorescence is observed in the PHB+ cells (PEA ∼ 10^4 a.u.) compared to PHB− cells (PEA < 10^3 a.u.), indicating the presence of PHB granules inside the cells that are stained more strongly than the lipid membranes.

After testing the method with *C. necator*, *E. coli* PHB+ and PHB− were examined for extracellular synthesis of PHB (Figure 3). Since the PHB granules were visualized outside the

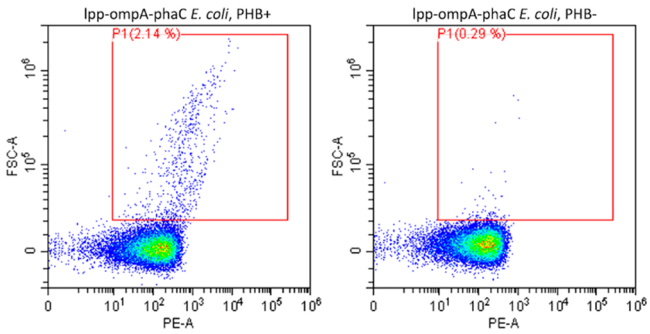

Figure 3. Flow cytometry analysis of Nile red-stained lpp-ompA-phaC *E. coli* cells with both PHB+ and PHB− treatments. Events are plotted with forward scattering absorbance (FSC-A) versus phycoerythrin fluorescence channel signal (PE-A, n = 30,000 events).

confines of the bacterial cell by microscopy, it was hypothesized the PHB granules would be detected by the flow cytometer as individual events, separate from the detected bacterial cells. The results were striking in that the majority of the events showed the same FSC pattern, likely indicating there were no changes in bacterial cell size. However, in the PHB+ sample, the amount of events diverging from the majority were roughly 7-fold (∼640 events) when compared to the PHB− sample (87 events), indicated by the selected event box P1. It is sensible that most of these P1 events represent the extracellular PHB granules. This is because compared to the majority of events (the bacterial cells) these P1 events demonstrated higher forward scattering (FSC > 10^5 a.u. compared to FSC < 10^4 a.u.), side scattering (SSC > 10^6 a.u. compared to SSC < 10^5 a.u., Figure S7), and fluorescence (PEA > 10^3 a.u. compared to PEA ∼ 10^2 a.u., Figure S8). These quantitative results are consistent with the qualitative findings that compared to bacterial cells, PHB granules are visualized as larger, more opaque, and more strongly dyed by Nile red, respectively.

CONCLUSION

Characterized chemically by ^{1}H NMR and FT-IR, polymer granules of PHB were synthesized by genetically engineered bacterial cells, and this was both qualitatively and quantitatively determined to be performed extracellularly. This work demonstrates a proof-of-concept that bioplastics can be biosynthesized extracellularly, providing a continuous, scalable

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process that is an improvement from current state-of-the-art manufacturing of PHB and other biomaterials. Future work must explore using more abundant precursors and reagents, as well as fundamental exploration of the surface-displayed enzyme structure and function. In addition, engineering strategies will need to be employed to improve the separation process to obtain pure products and materials without bacterial contaminants.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmaterialsau.3c00059.

Detailed methodology, additional ^{1}H NMR spectra for all samples, scanning electron microscopy images, transmission electron microscopy images, bright field fluorescence microscopy images, additional flow cytometry figures for *E. coli* and control PHB-producing strain, *Cupriavidus necator* (PDF)

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Author Contributions

K.B. and S.D.M. conceived the study and designed experimental setup. K.B. grew *E. coli* and biosynthesized PHB. K.B. characterized PHB synthesis with NMR, FT-IR, and flow cytometry. A.D. performed SEM, W.L performed TEM, and M.B. performed fluorescence microscopy on the samples. K.B. and S.D.M. critically analyzed the results and wrote the manuscript that was edited and approved from all authors.

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Notes

The authors declare no competing financial interest.

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