End-functionalization of poly(3-hydroxybutyrate) *via* genetic engineering for solid surface modification†

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Received (in Cambridge, MA, USA) 13th October 2004, Accepted 2nd February 2005
First published as an Advance Article on the web 18th February 2005
DOI: 10.1039/b415809a

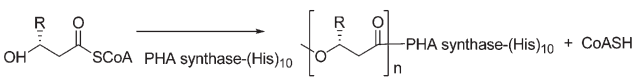
A new approach to end-functionalization of poly(3-hydroxybutyrate) [PHB] is described. Using genetically engineered PHB synthase fused with a 10x-histidine units at its N-terminus, end-functionalized PHB was synthesized and used for the solid surface modification.

End-functionalized polymers containing a reactive group on one or both ends are widely used in colloidal applications, catalysis, drug delivery systems, and surface modification.[1] Typical end-functionalization methods involve the use of functional initiators or the transformation of polymer chain ends.[2] While these methods can readily add a desired functional group to the end of a polymer chain, it is difficult to control the chain end sequence for more than one terminal unit. On the other hand, in living biological systems, the control of sequence at the end of the chain of polymers (*e.g.* proteins and nucleic acids) is extremely precise. Therefore, biological processes to control the functionality and sequence of polymer chain (end) has been of great interest in polymer science.[3,4] Here, we describe a new approach using a biological process to functionalize the end groups of poly(3-hydroxyalkanoate) [PHA].

PHAs are aliphatic polyesters produced by a variety of microorganisms,[5] and their properties span a wide range, from elastomers to thermoplastics, depending on their monomers. Therefore, PHAs are regarded as one of the most promising candidates to replace petrochemical-based commodity polymers. PHAs are also biodegradable and biocompatible polymers and have many potential biomedical applications.[6] In PHA-producing bacteria, PHA synthase is the key enzyme in PHA biosynthesis and catalyzes the conversion of hydroxyacyl-Coenzyme A substrates to PHAs. The unique feature of its enzymatic polymerization is that the reaction occurs through covalent catalysis; the synthesized polymer chain is covalently bound to a specific amino acid residue within the enzyme (Scheme 1).[7] We recently showed that the PHA synthase immobilized on silicon surface retained activity and synthesized PHA directly on the surface to form a stable polymeric layer.[8]

The covalent linkage between the enzyme and the polymer allows for the preparation of a block copolymer in which one block is composed of PHA synthase whose sequence can be modified *via* genetic engineering. This entails fusing the PHA synthase to a His-tag (10x-histidine) then expressing it in recombinant *E. coli*, followed by purification. The purified enzyme is subsequently used to synthesize PHB to produce a covalently-bound protein–polymer hybrid with His-tag end-functionality (Fig. 1). Our previous work confirmed that the resulting protein–polymer complex is highly stable, supporting the idea of covalent catalysis.[7,8] PHB is the most common homopolymer member of the PHA family and has a methyl group in its side chain (R group). The end-functionalized protein–polymer hybrid was able to complex onto a Ni^{2+}-nitrilotriacetic acid (Ni–NTA)-derivatized surface (silicon or agarose) as a result of the tight interaction between the $(His)_{10}$-tag and the Ni–NTA (Fig. 1).[9]

Addition of a poly(His)-tag to either the N- or C-terminus of a protein is widely used to facilitate protein purification using metal affinity chromatography. *E. coli* BL21 (DE3) pLysS was transformed with a plasmid containing a modified gene encoding His-tagged PHA synthase. After purification under native conditions, the modified enzyme retained normal catalytic activity.



Scheme 1 The formation of the PHA–protein complex using PHA synthase. The R group typically ranges from CH_3 [giving rise to PHB] to $(CH_2)_{10}CH_3$ [resulting in poly (3-hydroxybutadecanoate)].

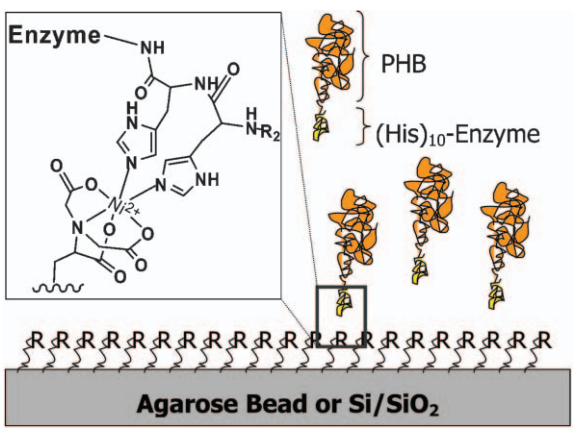


Fig. 1 Schematic representation of end-functionalized PHB block copolymers complexing onto a Ni–NTA derivatized solid surface. The inset shows a part of his-tag complexed with Ni–NTA on the surface. R represents Ni–NTA and R_2 for the rest of histidine units.

† Electronic supplementary information (ESI) available: detailed experimental procedures. See http://www.rsc.org/suppdata/cc/b4/b415809a/

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It has been reported that the N-terminus of PHA synthase is highly variable, and approximately 100 N-terminal amino acid residues can be removed without affecting the enzyme's activity.[10] Therefore, further modification of the N-terminus of the protein to confer alternative functionalities without affecting the protein's native catalytic activity was predictable.

In vitro synthesis of PHB was carried out with 3-hydroxybutyryl-CoA (3HB-CoA) as a substrate at room temperature. The substrate (5 m*M* 3HB-CoA) was consumed within 10 min using 0.5 μM enzyme. The synthesized PHB was collected by centrifugation, washed with methyl alcohol and dissolved in chloroform. Complexing the end-functionalized polymeric material onto Ni–NTA agarose beads (QIAGEN, Inc.) was carried out by immersing the beads into a solution containing *in vitro*-synthesized PHB (1 mg PHB in 1 mL chloroform) and incubating them with mild agitation at room temperature for 1 h. The Ni–NTA agarose was thoroughly washed with chloroform and stained with Nile red for visualization with a fluorescence microscope. Fluorescence micrographs of the stained PHB complexing onto an Ni–NTA agarose bead revealed that the entire surface was coated with end-functionalized PHB (Fig. 2b).

The protein–PHB hybrid was also complexed onto the patterned Ni–NTA-derivatized silicon surface. The preparation of the patterned Ni–NTA-derivatized silicon surface is described in detail in the supporting information†. The Ni–NTA derivatized silicon wafer was immersed into *in vitro* synthesized PHB solution (1 mg PHB in 1 mL chloroform), and incubated at room temperature for 1 h with mild agitation. The silicon surface was next thoroughly rinsed with chloroform and stained with a fluorescent dye, Nile red, for visualization. The silicon surface modified with Ni–NTA allowed stable complexation of the end-functionalized polymer (protein–PHB) onto the surface (Fig. 3b). Polymer-free His-tagged PHA synthase was also shown to stably complex to the same surface (Fig. 3a). Non-specific binding of His-tagged protein or protein–polymer complex to bare silicon surface was negligible; this result supported our conclusion that specific binding of end-functionalized polymers to the Ni–NTA derivitized surface was occurring.

Atomic force microscope (AFM) analysis revealed that Ni–NTA derivatized silicon surface was completely coated with protein–PHB complex with average thickness of 15 nm (Fig. 4b). The surface presented evenly distributed grain structures. The diameter of grains ranged from 30 to 50 nm. The thickness and morphology of the grain structures of this polymer film are different from the sample prepared by direct enzymatic surface-initiated polymerization (grafting from method); the average thicknesses of polymer film and grain diameter are 200 nm and

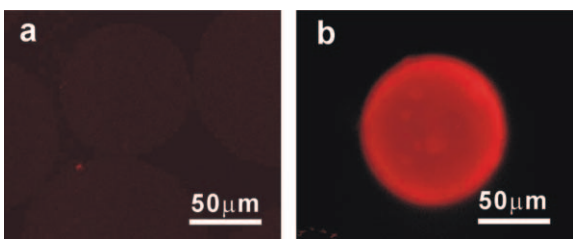

Fig. 2 Fluorescence micrograph of (a) a bare Ni–NTA agarose bead and (b) PHB-grafted Ni–NTA agarose bead after staining with Nile red.

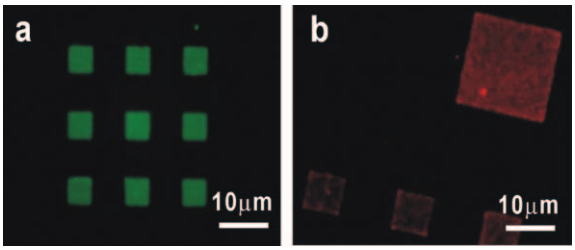

Fig. 3 (a) Fluorescence micrograph of polymer-free His-tagged PHA synthase immobilized onto a Ni–NTA-patterned silicon surface; labeled with FITC-conjugated antibody. (b) His-tagged protein–PHB hybrid complexed onto a Ni–NTA-patterned silicon surface; stained with Nile red for visualization.

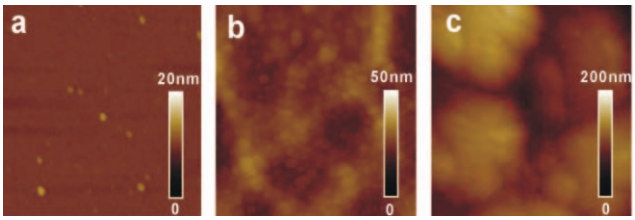

Fig. 4 AFM images of silicon surfaces after (a) derivatizing with Ni–NTA, (b) complexing His-tagged protein–PHB hybrid onto Ni–NTA derivatized silicon surface and (c) PHB was grown from Ni–NTA derivatized surface by immobilized enzyme. The field sizes of all the images are 1 μm × 1 μm.

600 nm, respectively (Fig. 4c).[8] We believe that this is a result of differences in density of polymer chains on the surface. In previous work, the polymer chains grow directly from the surface through immobilized initiator (*i.e.* PHA synthase). The growing polymer chain stretches away from the surface, thus resulting in dense and thicker polymer film. Steric hindrance of the grafted protein–polymer hybrid on the reactive surface would prevent additional polymer chains from diffusing through the film to the reactive sites on the surface, resulting in the formation of a thin layer of less dense polymer film. The AFM image suggests that each grain structure was derived from single protein–PHB complex. Assuming that the density of PHB is 1.285 g cm^{-3} (ref.11) and molecular weight of single PHB chain is 3 to 12 × 10^6 Da,[12] the calculated size of single PHB grain is around 20–30 nm, which is comparable to the domain size shown in Fig. 4b. The enzyme comprises only 2% of total molecular weight of the protein–polymer complex, and therefore its contribution to the grain size would be small.

In conclusion, we demonstrated a new approach to end-functionalize PHB using genetically engineered PHA synthase and its ability to modify solid surfaces. This work demonstrates that the modification of PHA end-groups by protein engineering enables the introduction of a wide variety of functionalities into PHAs that allow it to interact with specific ligands or receptors. We envision that this new approach will be a useful tool to develop novel classes of block copolymers of which one block is a member of the PHA family with potentially 100 different types of monomers[3,5] and the other block is protein with custom-designed sequences and functionalities.

This material is based upon work supported by the STC program of the National Science Foundation under Agreement

No. ECS-9876771 through the Nanobiotechnology Center of Cornell (NBTC).

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