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Tumor-specific hybrid polyhydroxybutyrate nanoparticle: Surface modification of nanoparticle by enzymatically synthesized functional block copolymer

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ABSTRACT

A new approach to functionalize the surface of hydrophobic nanocarrier through enzymatic polymerization was demonstrated. The effective coupling between the hydrophobic surface of PHB nanoparticle and PHB chain grown from the enzyme fused with a specific ligand provided a simple way of functionalizing nanoparticles with active protein layers in aqueous environment. PHB nanoparticles loaded with model drug molecule, Nile red, were prepared through oil-in-water emulsion solvent evaporation method and the surface of nanoparticles were functionalized with tumor-specific ligand, RGD4C, fused with PHA synthase that drove the coupling reaction. The functionalized PHB nanoparticles showed a specific affinity to MDA-MB 231 breast cancer cells indicating that the tumor-specific ligand, RGD4C, was effectively displayed on the surface of PHB nanoparticles through enzymatic modification and confers targeting capability on the drug carrier.

Polymeric nanoparticles have long been investigated as drug carriers and imaging agents.[1–3] Among many techniques in preparing nanoparticles, oil-in-water (o/w) emulsion method has attracted a great deal of attention because the processes are relatively simple and also suitable for mass production.[4] The ligands on the surface of nanocarrier improve delivery efficiency through specific molecular recognition of the carrier with unique marker molecules in target cells. The surface modification of nanoparticle has been directed mostly by covalent attachment of affinity molecules to its surface but its use is depending on the availability of amine or carboxylic acid groups on the surface of nanoparticles. With the increasing needs for a simple and effective methods in preparing functional nanoparticles with targeting capability a number of non-covalent approaches have been developed based on electrostatic or ligand–receptor binding interactions.[5–7] Herein we demonstrate a new approach to prepare polyhydroxyalkanoate (PHA) nanoparticles as a drug carrier with targeting ability through simple o/w emulsion method in combination with biological surface functionalization.

Polyhydroxyalkanoates (PHAs) are aliphatic polyesters produced by a wide range of microorganisms as intracellular carbon and energy storage compounds.[8] PHAs have been proposed as being a fifth physiologically important organic biopolymers along with polyisoprenoids, polypeptides, polysaccharides and polynucleotides.[9] PHAs have received significant interests from industry and academia because of its biocompatible and biodegradable properties with potential applications in drug delivery and biomedical applications.[10] A stable and efficient immobilization of a specific ligand to the surface of PHA nanoparticles is necessary for the production of targeted drug carrier system. Several proteins, such as phasin and substrate binding domain of PHA depolymerase, that are associated with the formation and metabolism of PHA granules in microorganisms were reported to have an affinity to PHA granules and used as a linker to attach ligand molecules to PHA particles by genetically fusing them with a specific ligand.[11,12] On the other hand, the unique catalytic characteristics of PHA synthase were also utilized to produce PHA micelles with a specific ligand on the surface.[13,14] PHA synthase catalyzes polymerization of 3-hydroxybutyryl-coenzyme A (3HB-CoA) to PHA and the synthesized polymer chain remains covalently bound to a specific amino acid residue within the enzyme.[15] The covalent linkage between the polymer chain and enzyme resulted in the formation of amphiphilic block copolymer, which self-assembles into micelle structure with protein domain facing toward surrounding aqueous environment. But it requires relatively expensive substrate and is not ideal for mass production at this stage.

Here, we coupled the enzymatic process with o/w emulsion method to produce surface functionalized nanocarrier in cost effective manner. PHAs have been produced by bacterial fermentation and commercially available with reasonable price.

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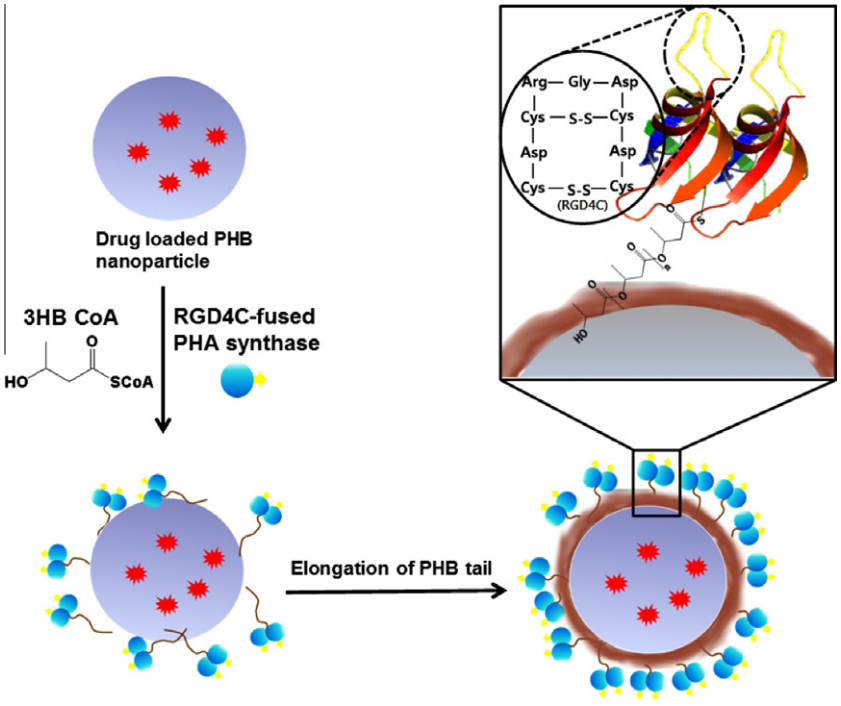
As shown in Scheme 1, PHA chains grown from the engineered PHA synthase interact with the surface of drug loaded PHA nanoparticle. The hydrophobic interaction between the surface of PHA nanoparticle and growing PHA chain from enzyme stabilized the core-shell structure. The RGD4C moiety that was co-expressed with PHA synthase as a fused form provided the carrier with a targeting capability toward cancer cells that overexpress integrin. To prepare RGD4C fused PHA synthase, A pair of oligonucleotides encoding RGD4C peptide was chemically synthesized and ligated into the upstream of *phaC* gene. The cloning, overexpression and purification of native and engineered PHA synthase was described in our previous study.[16]

Polyhydroxybutyrate (PHB) nanoparticles loaded with model drug molecule, Nile red, were prepared through oil-in-water emulsion solvent evaporation method as described in Supplementary data. Briefly, 50 mg PHB dissolved in 1 mL of chloroform along with 0.125 mg of Nile red was dispersed in 10 mL of 0.1% polyvinyl alcohol (PVA) under ultrasonication followed by solvent evaporation. The chloroform in oil phase was completely removed using rotary evaporator for 4 h at 30 °C under the pressure of 400 hPa. PHB nanoparticles were collected by centrifugation at 1500*g* for 5 min and washed with deionized water five times. Finally, the collected PHB nanoparticles were dispersed in 10 mL of deionized water with bath-sonication at 50 W for 5 min. The PHA nanoparticles were washed with distilled water three times on cellulose acetate filter paper (pore size of 200 nm) and dried in a vacuum chamber for further use. The diameter of the resulting PHB nanoparticles was determined to be 209 nm in average with a standard deviation of 59 nm from three independent experiments. Surface modification of PHB nanoparticles with targeting peptide, RGD4C, was carried out in 20 mM potassium phosphate buffer (pH 7.0) containing 100 mg of Nile red loaded PHB nanoparticle, 1.15 μM RGD4C fused PHB synthase, 5 mM 3-hydroxybutyryl-coenzyme A (3-HB-CoA) as a substrate and 200 mM NaCl with a total reaction volume of 100 μL. Upon the synthesis of PHB from 3-HB-CoA, the resulting protein–PHB hybrid molecules are driven to the hydrophobic surface of PHB nanoparticles through hydrophobic interaction between PHB nanoparticles and PHB chain grown from the fusion enzyme. The elongation of PHB chains by fusion enzymes on the surface of PHB nanoparticle continues until the reaction ran out of substrate, 3-HB-CoA. At the end of reaction, the PHB nanoparticles made by oil in water method were fully coated with protein–PHB block copolymer along with the targeting ligand, RGD4C, as shown in Scheme 1. The thioester linkage between PHA synthase and PHA chain grown from the enzyme was reported to be very stable. According to the previous report,[16] the thioester linkage remained intact even after refluxing the enzyme–polymer complex in chloroform for 3 days. Hydrolysis or aminolysis of the linkage through the reaction with thiol or lysine group in physiological condition could also be possible. But the presence of stable PHA synthase–polymer complex in cytoplasm of *Escherichia coli*[17] indicates that the hydrolysis of the linkage over the time scale of delivery in practice would be minimal.

The surface functionalized PHB nanoparticles are now stabilized and well dispersed in aqueous solution with hydrophilic protein blocks surrounding the nanoparticles. The resulting hybrid PHB nanoparticles were characterized by dynamic light scattering measurement (ELS-Z2, OTSUKA) and field emission scanning electron microscopy (FE-SEM) (Fig. 1).

We observed a shift in size distribution of PHB nanoparticles after the reaction. The diameter of PHB nanoparticles increased by about 46 nm through the enzymatic surface modification. It means that the enzymatic synthesis of PHB on the surface of PHB nanoparticle contributed to the formation of protein–polymer shell with a thickness of 23 nm. We presume that PHB chains are tightly associated with neighboring ones while they are growing on the surface of PHB nanoparticle. At the same time, the hydrophilic protein moiety at polymer–water interface stabilizes the hydrophobic nanoparticles during and after the reaction in aqueous solution.

To confirm that the specific coupling is through enzymatic polymerization on the surface of PHB particle, the hybrid particles were examined with fluorescence microscope (Fig. 2). For visualization, a fusion protein, PHA synthase–green fluorescence protein (GFP), was constructed by fusing two genes, $phaC_{Re}$ and *gfp*. The fusion gene was ligated into pET21a and the constructed plasmid vector was introduced into *E. coli* BL21 for overexpression. Transformed



Scheme 1. Surface functionalization of hydrophobic PHB nanoparticles through enzymatic reaction.

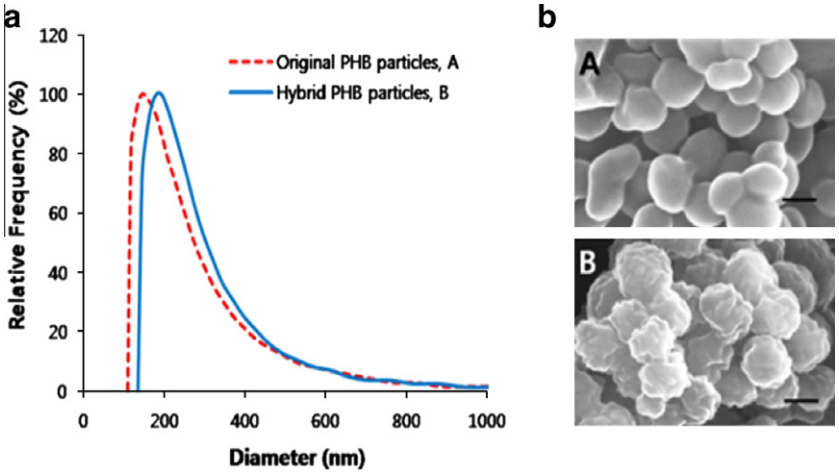


Figure 1. The DLS (a) and FE-SEM (b) analysis of native PHB nanoparticles versus surface functionalized hybrid PHB nanoparticles using RGD4C fused PHA synthase. (B) Scale bar is 100 nm.

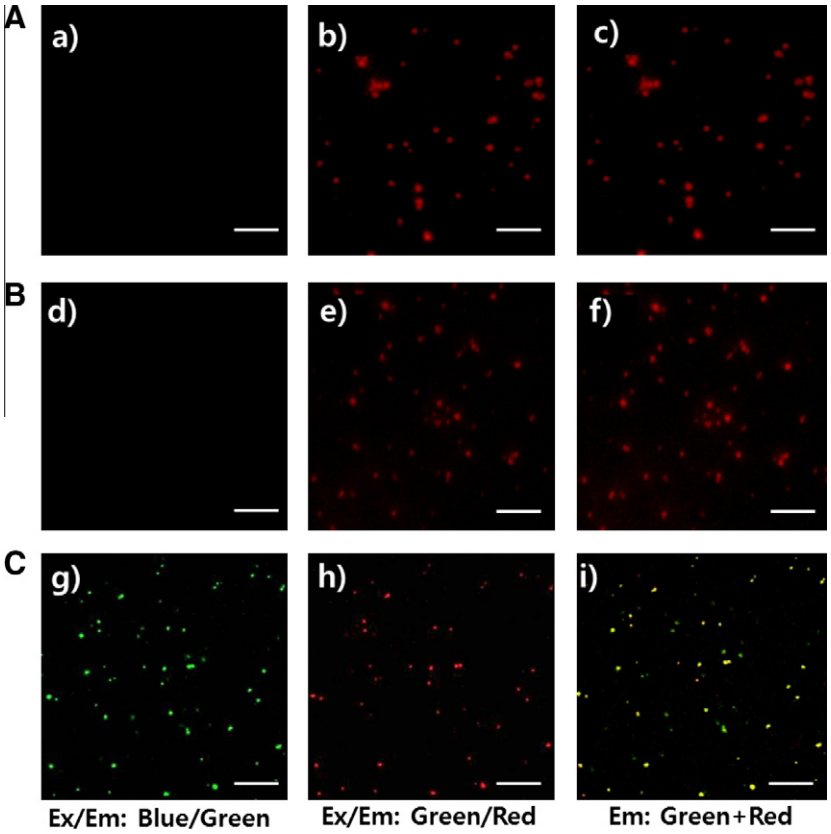


Figure 2. Fluorescence images of PHB microparticles reacted with (A) GFP, (B) GFP/PHA synthase fusion protein and (C) GFP/PHA synthase fusion protein and 3HB-CoA. The particles were examined for a green fluorescence (GFP), red fluorescence (PHB) and both (GFP + PHB). GFP is visible only when the fusion enzyme was added together with 3HB-CoA. Scale bar is 10 micron.

E. coli BL21 was grown aerobically at 37 °C with constant shaking in Luria Bertani (LB) medium containing ampicillin (100 μg/mL). Incubation of the culture continued to an optical density at 600 nm of 0.5. The culture was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and then grew at 18 °C with constant shaking at 200 rpm. After 16 h of induction, the cultures were harvested by centrifugation and the proteins were purified using a Ni-NTA agarose resin (QIAGEN) under native conditions according to the manufacturer's instructions. PHA synthase was expressed as a fused form with GFP at its N-terminus and both PHA synthase and GFP were active (Fig. S3). PHB microparticles were prepared with 1% Nile red. For this experiment, particles of micron scales were used for better visualization with optical microscope. Nile red is a lipophilic fluorescence dye molecule that is readily incorporated into the hydrophobic phase along with PHB during oil in water emulsification. PHB microparticles loaded with Nile red were subsequently treated with GFP, GFP-PHA synthase and GFP-PHA synthase with 3HB-CoA, respectively. After the reaction, the particles were washed with distilled

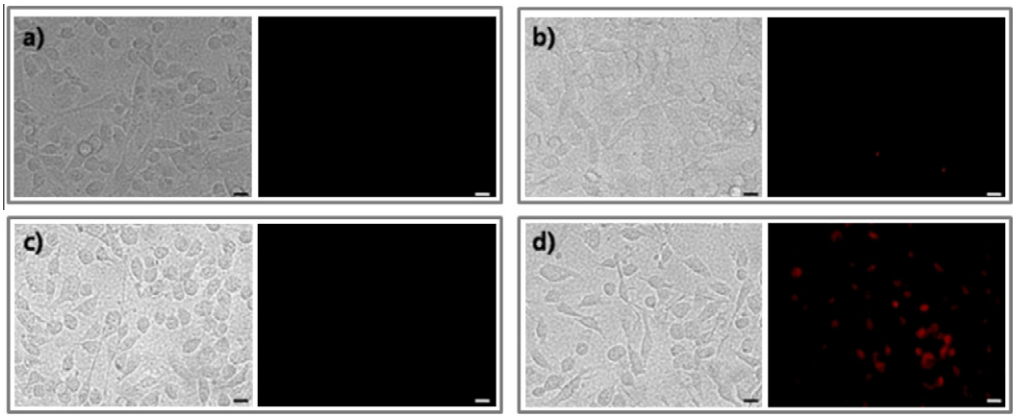


Figure 3. Bright field and fluorescence micrographs of MDA-MB-231 breast cancer cells after treatment with Nile red loaded PHB nanoparticles. (a) Control cells without any treatment,(b–d) cells treated with native PHB nanoparticles prepared by o/w emulsion method, (b) and hybrid PHA synthase prepared by enzymatic surface modification using native PHA synthase, (c) and RGD4C fused PHA synthase, (d) scale bar is 20 μm.

water and observed under fluorescence microscope. For the observation of Nile red loaded PHB particles, we used a green excitation filter block with excitation wavelength of 541–551 nm and emission wavelength of >580 nm. To visualize the signal from GFP, we used a GFP filter block with excitation wavelength of 450–490 nm and emission wavelength of 500–550 nm. The fluorescence image clearly shows PHB core in red color coated with GFP shell in green color (Fig 2C). It is evident that the coupling is not due to the non-specific binding of proteins, GFP or fusion protein, to the surface of PHB particles as shown in Figure 2A and B.

We tested targeting ability of the functionalized PHB nanoparticles for MDA-MB 231 breast cancer cells in vitro. MDA-MB 231 cells are human breast cancer cells that constitutively express $\alpha_v\beta_3$ integrins.[18–20] For targeting experiment, PHB nanoparticles were prepared with 1% Nile red. PHB nanoparticles loaded with Nile red were subsequently modified by enzymatic process described above using PHA synthase fused with RGD4C peptide. The adhesion and uptake of RGD4C-functionalized PHB nanoparticles by MDA-MB 231 cells were monitored with fluorescence microscope. MDA-MB 231 cells were planted into flat-bottom 96-well plates at a density of 5×10^4 cells per well. After 24 h of incubation under 5% CO_2, the cells were treated with functionalized PHB nanoparticles for 15 min at 37 °C. Unbound PHB nanoparticles were removed by washing with PHB buffer three times. The cells were imaged using a Nikon TE2000 inverted fluorescence microscope. The functionalized PHB nanoparticle emits red color upon excitation with green light (530 nm) due to Nile red molecules embedded in the core of PHB nanoparticles. The fluorescence microscopy revealed that the functionalized PHB nanoparticles are effectively bound to the target cells (Fig 3). On the other hand, the interaction between bare PHB nanoparticle and the target cells was negligible. The PHB nanoparticles processed with native PHA synthase did not show target specificity toward tumor cells. These results clearly showed that the tumor-specific ligand, RGD4C, is effectively displayed on the surface of PHB nanoparticle through enzymatic modification and confers targeting capability on the drug carrier.

In summary, we demonstrated a new approach to prepare a carrier system with targeting capability for imaging and drug delivery to cancer cells by integrating the unique catalytic characteristics of PHA synthase with simple oil in water emulsion methods. The effective coupling between the hydrophobic surface of PHB nanoparticle and PHB chain grown from the fusion enzyme provided a new way of functionalizing nanoparticles in economic way. We anticipate that this process will also be compatible with other hydrophobic seed nanoparticles. The immunological issue that might arise from the foreign protein on the surface of carrier remains to be cleared out by careful in vivo study. However this new approach provides very attractive means of designing nanostructures with multi-layers of active proteins in highly controlled manner. The catalytic activity of PHA synthase is not affected by attaching larger protein domain to either N-terminus or C-terminus. It will allow us to design a number of PHA synthases fused with variety of specific ligands, which would expand the application of this approach for the preparation of functionalized nanoparticles.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.03.058.

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