Electronic Supplementary Information (ESI)

Enzymatic synthesis of a drug delivery system based on polyhydroxyalkanoate-protein block copolymers

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Materials and Methods

Materials: A cloning vector, pET 15b, was purchased from Novagen (Novagen, Madison, WI, US). *Escherichia coli* DH5α (HIT^{TM}- DH5α, RBC, Taipei, Taiwan) and *Escherichia coli* BL21 (HIT^{TM}-21, RBC, Taipei, Taiwan) were used for cloning and expression of native and engineered PHA synthase, respectively. *Ralstonia eutropha* H16 was kindly provided by Dr. Carl Batt at Cornell University. Breast cancer cell line, MDA-MB-231 (Korean Cell Line Bank, Seoul, Korea) was used as an *in-vitro* targeting model. DL-*β*-Hydroxybutyryl coenzyme A, a substrate for enzymatic synthesis of PHB-protein copolymer, was purchased from Sigma-Aldrich (St. Louis, MO, US)

Preparation of native and RGD4C-fused PHA synthase:

phbC (PHA synthase gene) of *Ralstonia eutropha* H16 was amplified by polymerase chain reaction (PCR) with a primer set (forward primer: gagaagcatatgatcgagaatccaatc; reverse primer: ccgctcgagtgccttggctttgacgtat), and the purified PCR product was digested with *BamH*I and *Eco*RI and ligated into the corresponding sites of pET-19b (Novagen, Madison, WI, US). The purified PCR product amplified with a different set of primers (forward primer: gagaagcatatgatcgagaatccaatc; reverse primer: ccgctcgagtgccttggctttgacgtat) was digested with *Nde*I and *Xho*I, and ligated into the corresponding sites of pET-15b (Fig. S1-A). A set of complementary oligonucleotides (forward: tc gag gcg tgc gat tgc cgt ggc gat tgc ttc tgc ggc tga g; reverse: ga tcc tca gcc gca gaa gca atc gcc acg gca atc gca cgc c) encoding RGD4C motif was chemically synthesized (Bioneer Co, Daejeon, Korea), digested with *Xho*I and *BamH*I, and ligated into the downstream (carboxyl terminus) of *phbC* gene in pET15b vector (Fig. S1-B). In order to place the RGD4C motif at amino terminus of PHA synthase, another set of complementary oligonucleotides (forward: g gaa ttc cat atg gcg tgc gat tgc cgt ggc gat tgc ttc tgc ggc ctc gag cgg; reverse: ccg ctc gag gcc gca gaa gca atc gcc acg gca atc gca cgc cat atg gaa ttc g) encoding RGD4C motif was chemically synthesized (Bioneer Co, Daejeon, Korea), digested

with *Nde*I and *Xho*I, and ligated into the upstream (amino terminus) of *phbC* gene in pET-19b vector (Fig. S1-C).

For the expression of native and engineered PHA synthases, *E.coli* BL21 was transformed with pET-15b/*phbC*, pET-15b/*phbC*-RGD4C and pET-19b/RGD4C-*phbC*, respectively. Culture (100 ml) of transformed *E.coli* BL21 was grown aerobically at 37 °C with constant shaking in Luria Bertani (LB) medium containing ampicillin (100 μg/ml) to an optical density at 600nm of 0.6. The culture was then induced by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and then grown further at 18 °C with constant shaking at 200 rpm. After 4 hours of induction, the culture was harvested by centrifugation. PHA synthases were purified using a Ni-NTA agarose resin (Qiagen, Valencia, CA) under native conditions according to the manufacturer's instructions. Protein concentration was determined by the Bradford method with bovine serum albumin as a standard.[1] Size of purified enzymes was confirmed by 10 % SDS-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining.

Enzyme activity of native and RGD4C-fused enzymes:

The polymerization reaction was carried out in 20 mM potassium phosphate buffer (pH 7.0) containing 200 mM NaCl, 5 mM 3HB-CoA, and 4.6 μM enzymes at room temperature. The native PHA synthase, engineered PHA synthase fused with RGD4C at the amino terminal and carboxyl terminal with the same concentration were used to determine the enzyme activities. PHA synthase activities were determined by Ellman's assay that measures the free thiols released during the polymerization reaction.[2] A 10-μl aliquot of the reaction mixture was taken at every 2 min and mixed with 390 μl of 0.5 mM sodium acetate buffer (pH 4.7) containing 50 mM NaCl and 0.5 mM ethylenediaminetetraacetic acid (EDTA). A total of 690 μl of 40 mM sodium phosphate buffer (pH 7.6) containing 2 mM EDTA and 10 μl of 100 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were

subsequently added and mixed at room temperature for 1 min. Absorbance of the solution was spectrophotometrically measured at 412 nm using water as control. The molar extinction coefficient (cm^{-1} M^{-1}) of DTNB in reacting with free thiol is 13,600.

Preparation of PHB nanocarriers with native PHA synthase and engineered PHA synthase fused with RGD4C:

The reaction for the synthesis of PHA nanocarrier was carried out in 20 mM potassium phosphate buffer (pH 7.0) containing 200 mM NaCl, 5 mM 3HB-CoA and 4.6 μM enzymes with a total reaction volume of 100 μl at room temperature. For the targeting experiment, PHB nanocarriers were synthesized by engineered PHA synthases with 1% Nile red (0.15 mg Nile red/mL in dimethylsulfoxide) in the same reaction mixture. The excitation and emission wavelength of Nile red are 515-530 nm and 525-605 nm, respectively. After the reaction, excess Nile red were removed and the synthesized nanocarriers were collected by centrifugation at 10,000 rpm for 10 min followed by washing with RPMI 1640 medium five times.

Characterizations of PHB nanocarriers generated with native PHA synthase and engineered PHA synthase fused with RGD4C:

Synthesized PHB nanocarriers by native and engineered PHA synthases were analyzed by dynamic light scattering (ELS-Z2, OTSUKA, Japan) with laser wavelength of 638 nm and a scattering angle of 165°, and field emission scanning electron microscope (LEO supra 55, Carl zeiss, Germany) at 5 kV.

Tumor cell targeting assay:

MDA-MB-231 breast cancer cells were cultured in RPMI 1640 medium (Welgene, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and

streptomycin (100 mg/ml) at 37 °C in humidified atmosphere of 5% CO_2. In order to investigate the *in-vitro* targeting ability of ligand-conjugated PHB nanoparticles, trypsinized MDA-MB-231 cells were resuspended in serum-free media and plated into flat-bottom 96-well plates at density of 5×10^4 cells per well. After 24 hrs of incubation, the samples were added into the cell cultures. The treated MDA-MB-231 cells were incubated for 15 min in a CO_2 incubator 37 °C and unbound particles were removed by careful washing with PBS three times. Then, the cells were observed under the fluorescence microscope.

Supplementary figure

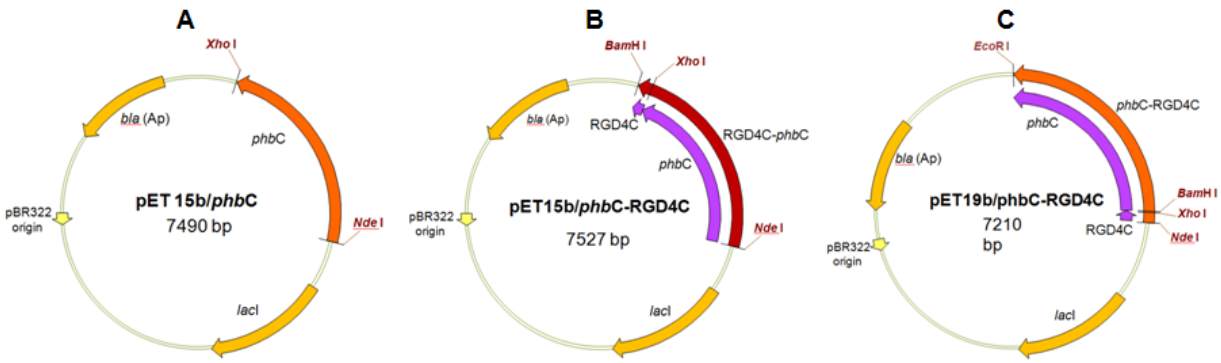

Figure S1. The map of three constructs for the expression of native PHA synthase (A), PHA synthase with RGD4C at carboxyl terminus (B), and PHA synthase with RGD4C at amino terminus (C), respectively.

References

1. M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248.
2. R. Singh, W. A. Blattler and A. R. Collinson, *Anal. Biochem.*, 1993, **213**, 49.