# Supporting Information

## Self-assembled multimeric-enzyme nanoreactor for robust and efficient biocatalysis

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**Experimental Section**

**Genes, plasmids and regents.** The pET24a-P450BM3 and the pET22b-GDH plasmids containing the *Bacillus megaterium* P450BM3 monooxygenase gene and *Bacillus subtilis* glucose dehydrogenase (GDH) gene were kindly provided by professor Byung-Gee Kim (Seoul National University, Korea) and professor Bingfang He (Nanjing University of Technology,China), repectively. Both the encoding sequences of SpyCatcher with a short peptide linker of $(GGGGS)_2$ and SpyTag with a linker-$(AGAGAGPEG)_5$ were synthesized by Genwiz (suzhou, china).The amino acid sequence of model enzymes and other proteins used in this research were listed in Table S2 at the end of supporting information. PrimeSTAR Max DNA polymerase, restriction endonucleases, and polymerase chain reaction (PCR) reagents were purchased from TaKaRa (Dalian, China). A protein assay kit was purchased from sangon (Beijing, China). The antibiotic and Ni-nitrilotriacetate (NTA), size exclusion chromatography column were from GE Health (Uppsala, Sweden). All other chemicals and reagents were analytical grade from Sigma (Sigma-Aldrich, USA).

**Construction of fusion genes.** The P450BM3m (**A74G/F87V/L188Q**) was obtained by using Quick change PCR by using the pET24a-P450BM3 as template and primers A74G FP/RP,F87V FP/RP and L188Q FP/RP. The Spycatcher-$(GGGGS)_2$ gene with 5' NcoI and 3' BamHI restriction sites, P450BM3m gene with 5'BamHI and 3' XhoI restriction sites were obtained from the pUC57-Spycatcher-$(GGGGS)_2$ and pET24a-P450BM3m by endonucleases digestion,then sequentially inserted into the pET28a, producing the pET28a-Spycatcher-P450BM3m. The pET28a-GDH was constructed by using a pET22-GDH as PCR template and the primers GDH1 FP/RP. then SpyTag-$(AGAGAGPEG)_4$ fragment was inserted into upstream restriction sites NdeI/BamHI of pET28a-GDH plamid,producing the plamid pET28a-Spytag-GDH.The primers used in plasmid construction listed in table S1. All of the plasmids were verified by sequencing and then transformed into *E. coli* BL21 (DE3) for recombinant protein expression. Primers used in the research were listed in Table S1.

**Table S1. Primers used in the plasmid construciton**

| Gene | Primer | Sequences (5' to 3') |
|---|---|---|
| **P450BM3m (A74G/F87V/L188Q)** | A74G-FP | AAGTCAAGGTCTTAAATTTGTACGTGAT |
| | A74G-RP | ATTTAAGACCTTGACTTAAGTTTTTATCA |
| | F87V-FP | GACGGGTTAGTTACAAGCTGGACGCATG |
| | F87V-RP | GCTTGTAACTAACCCGTCTCCTGCAAAAT |
| | L188Q-FP | ATGAACAAGCAGCAGCGAGCAAATC |
| | L188Q-RP | TTGCTCGCTGCTGCTTGTTCATTGC |
| **GDH1(BamHI/XhoI)** | FP | ATGGTGGATCCATGTATCCGGATTTAAAAG |
| | RP | TTATACTCGAGTTAACCGCGGCCTGCCT |
| **GDH2(NdeI/XhoI)** | FP | GGTCCGCATATGTATCCGGATTTAAAAG |
| | RP | ATAATCTCGAGTTAACCGCGGCCTGCC |

Restriction enyzme sites are underlined

**Protein expression and purification**. Recombinant *E. coli* BL21 (DE3) strains were cultured in Luria broth (LB) containing 100 μg/ml kanamycin at 37°C. When the optical density at 600 nm reached 0.8, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and and cells were incubated at 20 °C for 16 h. then harvested by centrifugation at 8,000 rpm for 10 min and resuspended in 20 mM Tris•HCl buffer (pH 8.0). Resuspended cells were lysed by homogenizer at 4 ℃. After centrifugation at 12,000 rpm for 30 min, the supernatant was loaded into Ni-NTA agarose gravity-flow column, and then washed with the buffer consisting of 20 mM Tris•HCl (pH 8.0), 0.5 M NaCl, and 30 mM imidazole. Proteins were eluted by the buffer consisting of 20 mM Tris•HCl (pH 8.0), 0.5M NaCl and 250 mM imidazole. Elution fractions were desalted and concentrated. Size exclusion chromatography analysis were performed by using a Superdex200 increase 10/300 column on AKTA FPLC (GE Healthcare Life Sciences). The buffer of 20 mM Tris•HCl (pH 8.0), 0.05M NaCl was used while applying a flow rate of 0.5 mL/min and fractions were collected in 0.5 mL.

**The protein concentration exmaination.** Protein concentration of glucose dehydrogenase was determined by the BCA assay . Bovine Serum Albumin was used as standard protein. Concentrations of P450 BM3 and SpyCatcher-P450 BM3 were determined by measuring CO-difference spectra (Omura and Sato1964). After the addition of sodium dithionite (10mg), The samples were then bubbled with carbon monoxide ( ~ 90 s) and incubated for 1 min before the UV spectrum from 400 to 500 nm was recorded,the amount of P450 was calculated based on the maximum absorbance at 450 nm minus the absorption at 490 nm( $\varepsilon_{450nm-490nm} = 91\ mM^{-1}cm^{-1}$).[1]

**Enzymatic activity assay.** Activities of SpyCatcher-P450BM3m, SpyTag-GDH and non-fused counterparts were measured by monitoring the decrease or increase in absorbance at 340 nm. The standard assay for P450BM3m activity was conducted with
20 mM Tris.HCl (pH 8.0), 100 mM NaCl, 5 mM indole, and 100 μM NADPH. For GDH, it was conducted with 20 mM Tris.HCl (pH 8.0), 100 mM NaCl, 500 mM glucose and 1.0 mM $NADP^+$. All measurements were made in triplicate at 25 °C. Reaction rates were determined by dividing the slope (O.D. change in the 1st minute upon initiation) in molar extinction coefficient of NADPH, 6220 $M^{-1}$ $cm^{-1}$, and path length of 1.0 cm.

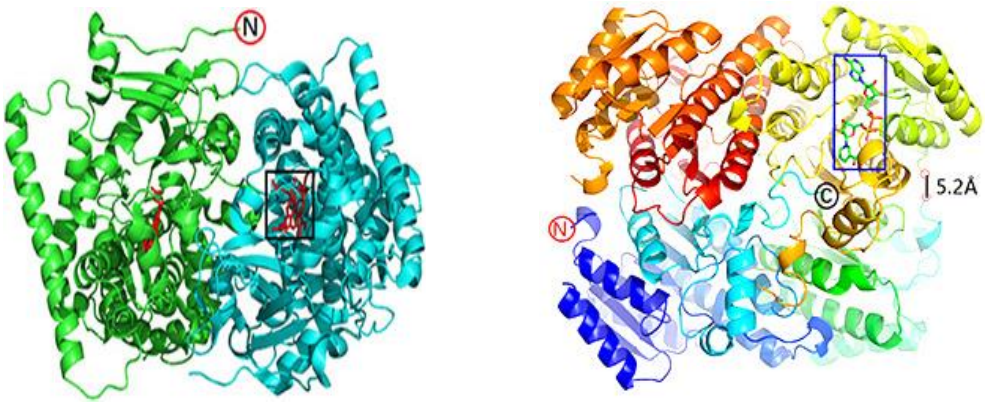
**Self-assembly and SDS-PAGE assay**. After determination of the protein concentrations, equal molar subunit of SpyCatcher-P450BM3m and SpyTag-GDH were mixed in the buffer containing 20 mM Tris.HCl (pH 8.0), 50 mM NaCl for 60 min at 4 ℃. The assemblies were subjected to 8.0 % SDS-PAGE for analysis.

**Dynamic light scattering assay.** Malvern nano zetasizer (Malvern,England) was used to measure and analyze SpyCatcher-P450BM3m, SpyTag-GDH and corresponding assemblies at various concentrations. Samples were centrifuged at 13000 rpm for 10 min prior to measure. The data were analysis by Zetasizer Software. All of the measurements were made in triplicate.

**FE-SEM, TEM and AFM assays.** For FE-SEM. P450-GDH MENCs samples were freeze-dried on a 10N lyophilizer(Christ, USA). Images were collected on an FEI Sirion 200 scanning electron microscope(FEI,USA) operated at 10 kV. To reduce charging effects, samples were sputter-coated with nanogold prior to analysis. For TEM, the samples were contrasted with 1.0% phosphotungstic acid. The TEM was carried out using a Tecnai G2 spirit Biotwin microscope (FEI ,USA). As for AFM, samples were prepared by dropping 10 μl of MENCs onto cleaved mica for 10 min, washing with deionized water, and drying under air. Images were collected in air using a scanning probe microscope (AFM/Multimode NanoscopeIIIa, Bruker, GER) operated in tapping mode. Image analysis was performed using the Nanoscope v.5.30 software package.

**Characterization of the P450BM3M-GDH MENCs**. The enzymatic activity for the synthesis of indigo was determined by monitoring the increase in absorbance at 670 nm, the extinction coefficient of indigo is 3.8mM $^{-1}$ cm $^{-1}$. This assay was carried out in the buffer of 20 mM Tris.HCl (pH 8.0), 100 mM NaCl , 500 mM glucose, 1.0 mM indole. The reaction system of the MENCs or enzyme controls were preincubated for 5 minutes, and then the reactions were initiated by adding various concentrations of $NADP^+$.

**The analysis of the stability and reusability.** The storage stability of P450BM3m-GDH MENCs and unassembled P450BM3m and GDH mixture were evaluated by measuring the residual enzymatic activity for the synthesis of indigo after storage in 20 mM Tris.HCl (pH 8.0) buffer after 1,3, 5, 7 and 10 days at 4 °C. The effect of temperature on MENCs and controls were determined by measuring the enzymatic activity before and after pre-incubation in 20 mM Tris•HCl buffer (pH 8.0) at temperatures ranging from 4 °C to 45 °C for 24 h. The initial activity was defined as 100%. The reusability of MENCs was investigated by multiple cycles ultrafiltration using a Amicon Ultra-15 Centrifugal Filter Device (MWCO 30kD, Millipore), and then analyzed their activities of synthesis of indigo. The residual activity of the MENCs after each cycle was normalized to the initial value.




A )　　B)

**Figure S1.** The quaternary structure of selected model enzymes. A) the structure of monooxygenage domain of P450BM3m (PDB:4RSN) from *Bacillus megaterium*, indicating the N terminus (in a red cycle) and heme binding sites (in a black rectangle). B) the structure of glucose dehydrogenase from *Bacillus subtilis*, which is homology modeled with *Bacillus megaterium* GDH (PDB:3AY6); the N-terminus (in a red cycle), C-terminus (in a black cycle) and cofactor $NADP^+$/NADPH binding sites (in a blue rectangle) were shown.

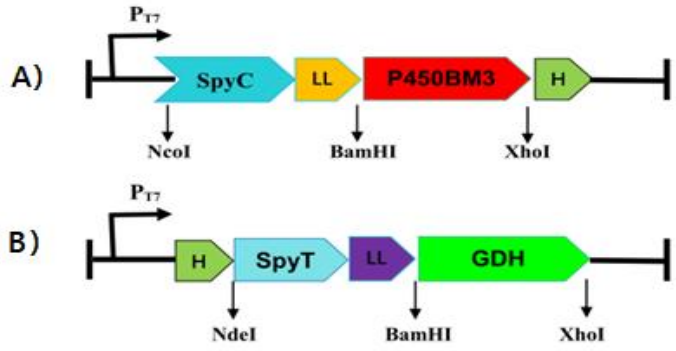



**Figure S2.** Constructed plasmid maps for expressing fusion proteins.

A) The pET28a-SpyCatcher-P450BM3m plasmid, the LL :the $(GGGGS)_2$ linker;

B) The pET28a-SpyTag-GDH plasmid, the LL repesents the $[(AG)_3PEG]_5$ linker, the SpyC: the SpyCatcher domain, the SpyT: SpyTag domain, the H: 6 *His tag;

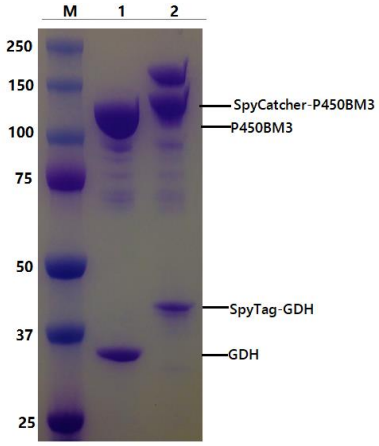



**Figure S3.** The SDS-PAGE analysis of assembled and unassembled P450BM3m-GDH. M, Protein markers (kDa). 1, P450BM3m (Caculated MW:119 kDa) and GDH (Caculated MW: 30 kDa). 2, SpyCatcher-P450BM3m (Caculated MW:131kDa) and SpyTag-GDH (Caculated MW: 35 kDa). Samples were prepared by mixing two enzymes at 2 μM subunit concentration, and then subjected to 10% SDS-PAGE analysis. The gels were stained with coomassie bright blue.

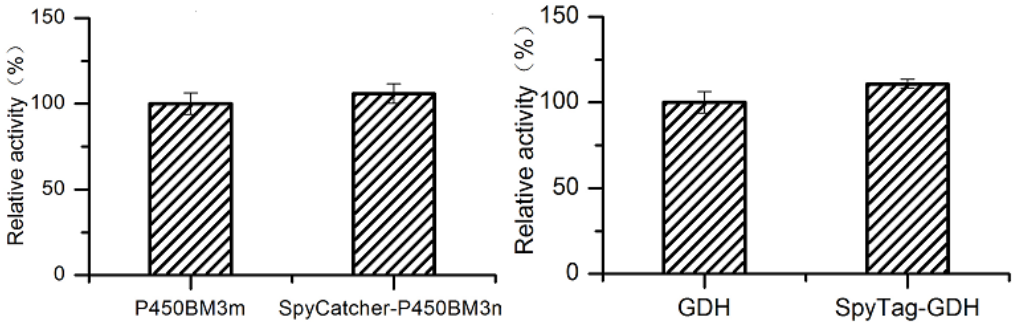



**Figure S4.** The relative enzymatic activity of constructed SpyCatcher-P450BM3m, SpyTag-GDH and nonfused enzymes. The activity of SpyCatcher-P450BM3m, SpyTag-GDH and nonfused counterparts were measured by examining the decrease (NADPH) or increase (NADP+) in absorbance at 340 nm, respectively. All measurements were conducted in triplicate.

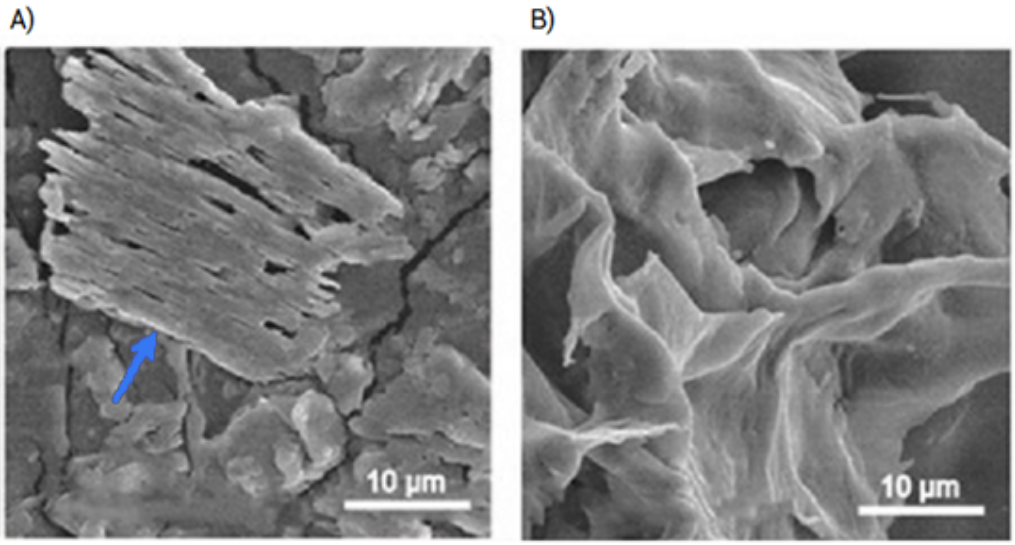



**Figure S5**. The representative SEM images of the supramolecular nanodevices. A) The soluble MENCs. B) Precipitated MENCs. Samples were prepared by mixing 2 µM SpyCatcher-P450BM3m and SpyTag-GDH subunits. The precipitated MENCs were separated from solution by centrifugation, then the MENCs were lyophilized and sputter-coated with nano gold prior to analysis. The blue arrow indicates the MENCs boundary.

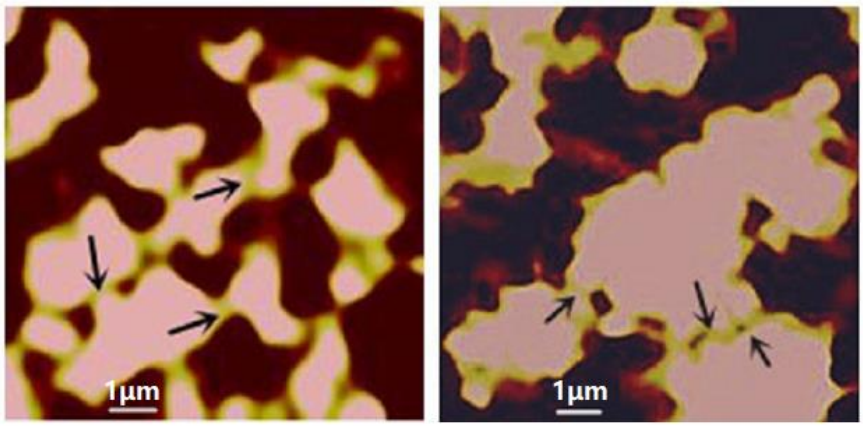



**Figure S6**. The representative AFM images of the MENCs. Samples were prepared by mixing 2 µM SpyCatcher-P450BM3m and SpyTag-GDH subunits. The black arrows indicate the the boundaries of MENCs.

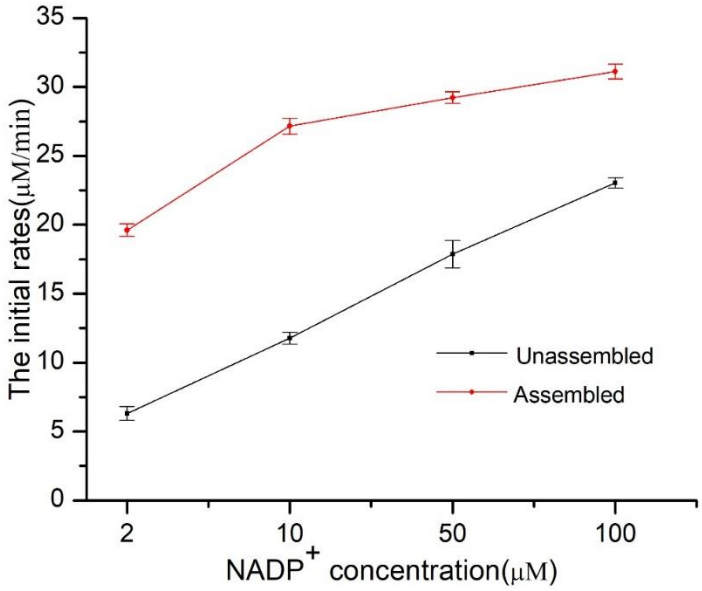



**Figure S7.** The initial reaction rates of the MENCs and the controls at various concentration of $NADP^+$. All measurements conducted in triplicate.

## Table S2. The amino acid sequences of proteins.

**P450BM3m**

MTIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELGEIFKFEAPGRVTRYLSSQRLIK
EACDESRFDKNLSQ**G**LKFVRDFAGDGL**V**TSWTHEKNWKKAHNILLPSFSQQAMKGYHAMM
VDIAVQLVQKWERLNADEHIEVPEDMTRLTLDTIGLCGFNYRFNSFYRDQPHPFITSMVR
ALDEAMNK**Q**QRANPDDPAYDENKRQFQEDIKVMNDLVDKIIADRKASGEQSDDLLTHMLN
GKDPETGEPLDDENIRYQIITFLIAGHETTSGLLSFALYFLVKNPHVLQKAAEEAARVLV
DPVPSYKQVKQLKYVGMVLNEALRLWPTAPAFSLYAKEDTVLGGEYPLEKGDELMVLIPQ
LHRDKTIWGDDVEEFRPERFENPSAIPQHAFKPFGNGQRACIGQQFALHEATLVLGMMLK
HFDFEDHTNYELDIKETLTLKPEGFVVKAKSKKIPLGGIPSPSTEQSAKKVRKKAENAHN
TPLLVLYGSNMGTAEGTARDLADIAMSKGFAPQVATLDSHAGNLPREGAVLIVTASYNGH
PPDNAKQFVDWLDQASADEVKGVRYSVFGCGDKNWATTYQKVPAFIDETLAAKGAENIAD
RGEADASDDFEGTYEEWREHMWSDVAAYFNLDIENSEDNKSTLSLQFVDSAADMPLAKMH
GAFSTNVVASKELQQPGSARSTRHLEIELPKEASYQEGDHLGVIPRNYEGIVNRVTARFG
LDASQQIRLEAEEEKLAHLPLAKTVSVEELLQYVELQDPVTRTQLRAMAAKTVCPPHKVE
LEALLEKQAYKEQVLAKRLTMLELLEKYPACEMKFSEFIALLPSIRPRYYSISSSPRVDE
KQASITVSVVSGEAWSGYGEYKGIASNYLAELQEGDTITCFISTPQSEFTLPKDPETPLI
MVGPGTGVAPFRGFVQARKQLKEQGQSLGEAHLYFGCRSPHEDYLYQEELENAQSEGIIT
LHTAFSRMPNQPKTYVQHVMEQDGKKLIEL LDQGAHFYICGDGSQMAPAVEATLMKSYAD
VHQVSEADARLWLQQLEEKGRYAKDVWAG

**GD**H

MYPDLKGKVVAITGAASGLGKAMAIRFGKEQAKVVINYYSNKQDPNEVKEEVIKAGGEAV
VVQGDVTKEEDVKNIVQTAIKEFGTLDIMINNAGLENPVPSHEMPLKDWDKVIGTNLTGA
FLGSREAIKYFVENDIKGNVINMSSVHEVIPWPLFVHYAASKGGIKLMTETLALEYAPKG
IRVNNIGPGAINTPINAEKFADPKQKADVESMIPMGYIGEPEEIAAVAAWLASKEASYVT
GITLFADGGMTQYPSFQAGRG

**SpyCatcher-GS linker**

HMAMVDTLSGLSSEQGQSGDMTIEEDSATHIKFSKRDEDGKELAGATMELRDSSGKTIST
WISDGQVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGKATKGDAHIGGG
GSGGGGS

**SpyTag-[(AG)$_3$PEG]$_5$ linker**

HMAHIVMVDAYKPTKGGSELAGAGAGPEGAGAGAGPEGAGAGAGPEGAGAGAGPEGAGAGAGPEGGGS

## REFERENCE:

[1] Beyer, N.; Kulig, J.K.; Bartsch A.; Hayes M.A.; Janssen D.B.; Fraaije M.W. P450BM3 fused to phosphite dehydrogenase allows phosphite-driven selective oxidations. *Appl. Microbiol. Biotechnol*. ***2017,*** *101:2319-2331*. DOI: 10.1007/s00253-016-7993-7.