Electronic Supplementary Information (ESI):

Modular Multi-enzyme Cascade Process Using Highly Stabilized Enzyme Microbeads

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Figure S1. Digital image of the EMBs

Figure S2. Production rate control by the number of the enzyme microbead (EMB)

1. Experimental Section

1.1. Materials

Eight-arm polyethyleneglycol amine (hexaglycerol) (amine-PEG) (MW ~10,000, Jenkem Technology, Allen, TX, USA), alginic acid sodium salt (Sigma-Aldrich, St. Louis, MO, USA), calcium chloride (Sigma-Aldrich), sodium citrate (Sigma-Aldrich), tetramethyl orthosilicate (TMOS) (Sigma-Aldrich), tetraethyl orthosilicate (Sigma-Aldrich), 3-aminopropyltriethoxysilane (APTES) (Sigma-Aldrich), glutaraldehyde (GA) (Sigma-Aldrich), *N, N*-dimethylformamide (99%, Sigma-Aldrich), *p*-nitrophenyl butyrate (Sigma-Aldrich), phosphoenolpyruvic acid monosodium salt hydrate (Shimaz), sodium pyruvate (Sigma-Aldrich), sodium phosphate monobasic (Sigma-Aldrich), sodium phosphate dibasic (Sigma-Aldrich), potassium phosphate monobasic (Sigma-Aldrich), potassium phosphate dibasic (Sigma-Aldrich), magnesium sulfate (Sigma-Aldrich), Dynabeads M-270 Amine (amine-magnetic beads) (Invitrogen, Carlsbad, CA, USA), Atto 488 protein labeling kit fluorescent labeling of thiol groups (Jena Bioscience), and Amicon Ultra-0.5 3K (Millipore) were purchased and used without any additional purification. Carboxyl esterase (E.C 3.1.1.1) from *Rhyzopus oryzae* was purchased from Sigma-Aldrich, and Pyruvate kinase from rabbit muscle (E,C, 2.7.1.40) and lactic dehydrogenase from rabbit muscle (E.C 1.1.1.27) were from Sigma-Aldrich. These enzymes are used for enzyme immobilization.

1.2 Preparation of magnetically separable branched-polymer/silica-shell hybrid microbeads

Amine-PEG (30 wt%), alginic acid salt (1 wt%), and amine-magnetic bead solution (5 wt%) were dissolved in distilled water and then sonicated for 30 min at 25 °C. The polymer-magnetic beads solution was mixed with GA (0.5 wt%) and then sonicated for 60 min at

25 °C. The solution was loaded into a 10 mL syringe. Then, electrospray was performed at 3–4 kV high voltage (ES series; Gamma High Voltage Research, Ormond Beach, FL, USA) with 2.5 mL h^{-1} of flow rate. The 30 gauge syringe needle was fixed vertically 5 cm above the surface of GA (0.5 wt%) containing calcium chloride solution (100 mM).

The obtained microbeads were stirred overnight at room temperature for hardening and cross-linking. The alginate-PEG microbeads were washed more than three times with distilled water. Finally, the branched-polymeric microbeads were produced following liquefaction of the Ca-alginate gel embedded in the cross-linked branched-polymer by incubating in sodium citrate solution (100 mM) as the calcium chelating agent for 15 min. The microbeads were washed three times with distilled water, and stored at 4 °C.

The magnetically separable branched-polymeric microbeads were coated with silica by the modified sol-gel process using APTES and TMOS to form the organic-inorganic hybrid microbeads. The microbead solutions were mixed with *n*-hexane and stirred for 2 min. Then, APTES and TMOS were added sequentially with 5 min interval. The volume ratio of H_2O: *n*-hexane: APTES: TMOS was 25:50:4:3. The branched-polymer/silica shell structures were shaken for 2 h and then washed in distilled water. The branched-polymer/silica shell hybrid microbeads were stored at 4 °C until enzyme immobilization.

1.3. Characterization of the synthesized magnetically separable branched-polymer/silica-shell hybrid microbeads

The morphologic and elemental analyses of the microbeads were investigated by scanning electron microscopy (SEM; S-2360N) equipped with energy-dispersive X-ray spectroscopy, Hitachi Co. Ltd., Tokyo, Japan). Fourier transform infrared (FT-IR) spectra of the microbeads were measured on an Infinity gold FT-IR spectrometer (Thermomattson. Co. Ltd., Madison, WI, USA) using the ATR accessory.

1.4. Preparation of enzyme microbead (EMB)

To prepare the CE-EMBs, CE solution was prepared at 10 mg mL^{-1} in the sodium phosphate buffer (100 mM, pH 8.0). The 200 of PEG/silica shell hybrid microbeads were mixed with the enzyme solution (1 mL) and incubated with 200 rpm at 25 °C for 30 min and 60 rpm at 4 °C for 2 h. Then, the enzyme was aggregated by adding the 2 mL solution containing GA (1.0 wt%) and ammonium sulfate (0.5 g), and incubate with 200 rpm at 25 °C for 2 h, and on the rocker (30 rpm) at 4 °C overnight. Finally, the EMBs were washed with sodium phosphate buffer (100 mM, pH 8.0); the remaining unbound functional groups on the GA were capped with Tris-HCl (100 mM, pH 7.8). After capping, CE-EMBs were washed three times with sodium phosphate buffer (100 mM, pH 6.5 and stored at 4 °C until further use.

Pyruvate kinase (PK) and lactic dehydrogenase (LDH) were immobilized in order to realize synthetic pathway reaction which converts phosphoenolpyruvate (PEP) to lactate. Pyruvate kinase or lactic dehydrogenase (10 mg mL^{-1} each) were suspended in sodium phosphate buffer (100 mM, pH 8.0). The 1000 of PEG /silica shell hybrid microbeads were soaked into the enzyme solutions (5 mL) and incubated at 200 at 25 °C for 30 min and 60 rpm at 4 °C for 2 h. The enzymes were aggregated by adding 10 mL solution containing GA (0.25 wt%) and ammonium sulfate (0.25 g mL^{-1}) and incubate with 200 rpm at 25 °C for 2 h, and on the rocker (30 rpm) at 4 °C overnight. Even though the specific activity of enzyme after GA treatment was decreased, the specific activity of enzyme immobilized and treated with GA on microbeads were shown to enhance the reusability and working stability (**Table S2**).

Table S2. Specific activities of the free and immobilized enzymes

Enzyme	Specific activity (free enzyme)	Specific activity (Immobilized)
Carboxyl esterase	79.00 mM/ min g	9.38 mM/min g
Pyruvate kinase	2.34 X 10^5 mM/ min g	6.01 X 10^2 mM/min g
Lactic dehydrogenase	1.30 X 10^5 mM/ min g	7.56 X 10^2 mM/ min g

Finally, the EMBs were washed with sodium phosphate buffer (100 mM, pH 8.0); the remaining unbound functional groups on the GA were capped with Tris-HCl (100 mM, pH 7.8). After capping, the EMBs were washed three times with potassium phosphate buffer (100 mM, pH 7.6) and stored at 4 °C until further use. The amounts of the immobilized enzymes (**Table S1**) were indirectly estimated by subtracting the protein concentrations in the remained solutions before and after the immobilization. Protein concentrations were measured by commercially available BCA protein assay kit with the instruction provided.

Table S1. Enzyme loading amount; the absolute activity of each free enzyme was reduced after the immobilization.

Enzyme	Loading amount per bead	Loading efficiency (loaded enzyme/ free enzyme)
Carboxyl esterase	2.6 μg / bead	26 %
Pyruvate kinase	1.3 μg / bead	13 %
Lactic dehydrogenase	1.4 μg / bead	14 %

1.5. Fluorescence labeling and immobilization of the enzymes for Confocal Laser Scanning Microscope (CLSM) image of the EMBs

CLSM was employed in order to clarify the location of the enzymes in the EMBs. PK and LDH were fluorescently labeled with Atto 488 dye in order to visualize the enzymes. The dye

was selected to be bound to thiol group, for amine group, the other reactive functional group in enzymes, would be used for the cross-linking reaction. The labeling process followed the protocol in the labeling kit. In brief, the first, enzyme solution (10 mg mL^{-1}) suspended in sodium phosphate buffer (100 mM, pH 7.2) was prepared. And 3 v/v% of of Tris-(2-carboxyethyl)phosphine hydrocholoride (TCEP) solution (10 mg mL^{-1}) was added into the enzyme solution and incubated for 15 min in 200 rpm shaking condition at 20 °C. Then, 39 v/v% of Atto 488 Maleimide suspended in DMF (10 mg mL^{-1}) was treated and incubated for 2 h in the 200 rpm shaking incubator at 20 °C. After that, 1.7 v/v% of Glutathione suspended in pure water (10 mg mL^{-1}) was mixed to the solution and incubated for 15 min, in 200 rpm shaking condition, at 20 °C. The last, the enzymes labeled was purified by Amicon Ultra-0.5 3K, a centrifugal filter which has 3000 NMWL (nominal molecular weight limit) and diluted with sodium phosphate buffer (100 mM, pH 8.0) to make up the concentration of 10 mg mL^{-1}. The enzymes labeled by fluorescence were immobilized just as the same process of the section of '*Preparation of the enzyme microbead (EMB)*' described above. The fluorescence labeled EMBs were scanned by CLSM (LSM-700, Carl Zeiss, Oberkochen, Germany).

1.6. Production rate control by the number of enzyme microbead (EMB)

The activity of the EMBs was checked by monitoring the production of *p*-nitrophenol from the hydrolysis of *p*-nitrophenyl butyrate dissolved in *N, N*-dimethylformamide. Briefly, phosphate buffer (5.0 mL of 100 mM, pH 6.5) containing *p*-nitrophenyl butyrate dissolved in *N, N*-dimethylformamide (50 μL of 50 mM) was prepared as the substrate. The 10, 30, 50, 70, and 90 CE-EMBs were dispersed in this substrate solution and shaken at 200 rpm. The initial activity was calculated from the change of absorbance at 400 nm for a minute, and the absorbance was equated with the *p*-nitrophenol concentration. One unit (U) of carboxyl

esterase activity was defined as the amount of enzyme releasing *p*-nitrophenol (1 μmol) per minute under the assay conditions.

The reaction activity of the PK-EMBs was determined as pyruvate production from PEP and the LDH-EMBs as lactate production from pyruvate. The production rates were defined as the releasing product concentration in the fixed 15 min reaction of each EMBs with their substrates and cofactors. In brief, for the PK-EMBs, 6 mL of potassium phosphate buffer (65 mM pH 7.6) containing $MgSO_4$ (6 mM), ADP (1.2 mM), NADH (1 mM), and LDH (1.2 U) was prepared. Different numbers (25, 50, 100, 150, and 200) of PK-EMBs were dispersed in the solution before the reaction. The reaction started as inputting PEP (1.0 mM) into the solution. The reaction took place in the 200 rpm shaking incubator at 25 °C. The increase of pyruvate was determined as indirect measurement of the decrease of the NADH concentration, which derived from the pyruvate production and its linked reaction to lactate production through the LDH reaction. The 340 nm absorbance was measured by spectrophotometer every 3 min for 15 min. The decrease of the absorbance was equated to the increase of the pyruvate production.

Similarly, for the LDH-EMBs, 6 mL of potassium phosphate buffer (65 mM pH 7.6) containing $MgSO_4$ (6 mM), and NADH (1 mM) was prepared. Different numbers (25, 50, 100, 150, and 200) of LDH-EMBs were dispersed in the substrate solution before the reaction. The reaction started as treating pyruvate (1.0 mM) into the solution. The reaction took place in the 200 rpm shaking incubate at 25 °C. The increase of lactate was determined as indirect measurement of the decrease of the NADH concentration, which was connected to pyruvate production. The 340 nm absorbance was measured by spectrophotometer every 3 min for 15 min. The decrease of the absorbance was equated to the increase of the lactate production.

All samples were tested in triplicate for an error analysis. The standard deviations were shown as error bars on the graphs.

1.7. Stability measurement of the EMBs

The 200 of CE-EMBs were reacted as the same method of the section of '1.6. *Production rate control by the number of enzyme microbead (EMB)*' described above. Then, the reacted EMBs were washed with phosphate buffer (100 mM, pH 6.5) three times for the stability experiments and stored at room temperature under shaking condition (200 rpm) for further use. The stability measurement was continued till certain time.

1.8. EMB-based modular in-vitro cascade reaction system

The modular *in-vitro* enzyme reactions with various numbers of the EMB were performed in order to confirm that the reaction rates were controlled by the number of the homogeneous EMBs. The reaction was determined as lactate production from PEP via pyruvate by sequential reaction of immobilized pyruvate kinase and lactic dehydrogenase. The 20 mL glass vials containing 100, 50, and 0 PK-EMB and 200, 100, and 0 LDH-EMB were prepared. The cascade reactions were performed with the combination of the 2 different enzyme vessels (**Figure 5**aii). The production rate was defined as the concentration of the released product in the fixed 15 min reaction of PK-EMB and another independent reaction of LDH-EMB as a cascade reaction. In brief, The 6 mL of potassium phosphate buffer (65 mM, pH 7.6) containing $MgSO_4$ (6 mM), ADP (1.2 mM), and NADH (1 mM) was prepared. The 200 of PK-EMB was dispersed in the solution. The reaction was started as inputting the PEP in to the vial to shaking incubator (200 rpm, 25 °C). After 15 min of reaction, the PK-EMBs were separated by magnet and whole solution was moved to the next vial in which the LDH-EMBs were prepared. As soon as the solution was moved, the vial was placed in the shaking incubator (200 rpm, 25 °C). The increase of lactate was determined as indirect measurement of the decrease of the NADH concentration; the 340 nm absorbance was measured by spectrophotometer.

All measurements were performed in triplicate. The standard deviations were shown as error bars on the graphs.

1.9. Stability measurement of the EMB-based modular in-vitro cascade reaction system

The repeated reaction of the modular *in-vitro* enzyme system was performed. The reaction methods were the same as the section of '*EMB-based modular in-vitro cascade reaction system*' described above. The EMBs after each reaction were washed with potassium phosphate buffer (100 mM, pH 7.6) three times for the next combination reactions. Total 9 sorts of the combination reactions were designated as a cycle (**Figure 5**c). Total 3 cycles were performed repeatedly.

2. Experimental Figures

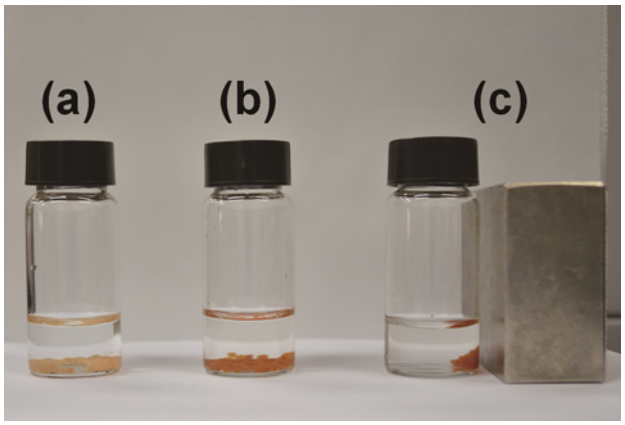

Figure S1. Digital image of the EMBs**;** (a) Organic/inorganic hybrid microbeads; (b) The EMBs turned to brown because of the imine groups which were newly formed after the enzyme immobilization; (c) The EMBs were separated within 2 seconds by magnetic force.

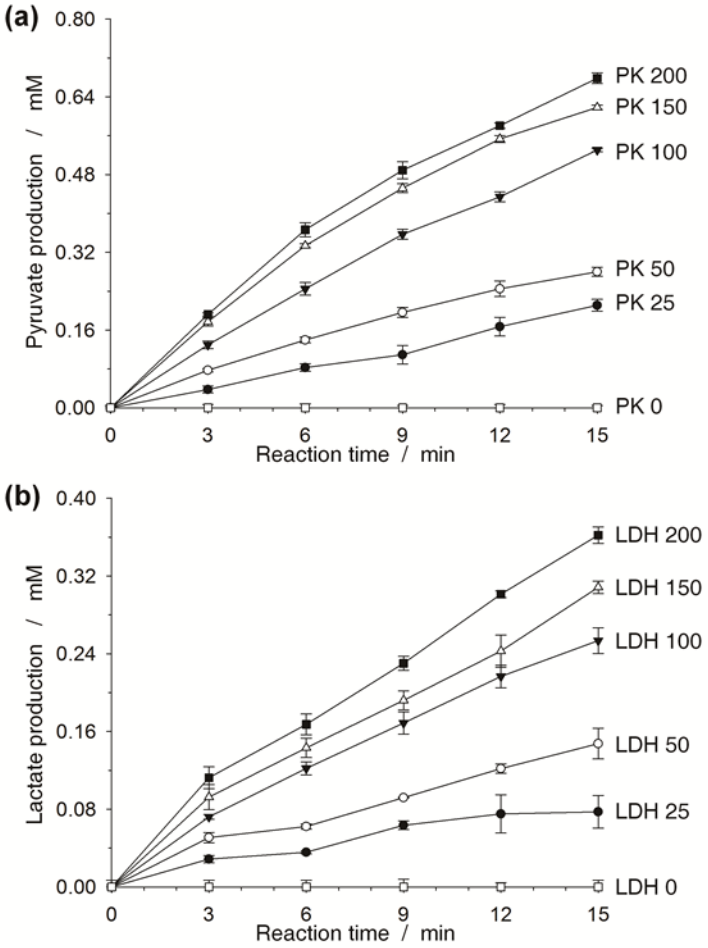

Figure S2. Production rate control by the number of the EMB**;** Additive effects of the EMB for increasing the production rate by increasing the initial activity of the enzyme reaction. 0, 25 50, 100, 150, and 200 EMB was used for each sort of the EMB; (a) PK-EMB; (b) LDH-EMB