Electronic Supplementary Information (ESI)

Enzyme-encapsulated microreactor for efficient theanine synthesis

Shun-ichi Matsuura,*[a] Takuji Yokoyama,[b] Ryo Ishii,[a] Tetsuji Itoh,[a] Emiko Tomon,[a] Satoshi Hamakawa,[a] Tatsuo Tsunoda,[a] Fujio Mizukami,[a] Hironobu Nanbu[b] and Taka-aki Hanaoka[a]

[a] *Research Center for Compact Chemical System, National Institute of Advanced Industrial Science and Technology (AIST), Nigatake 4-2-1, Miyagino-ku, Sendai 983-8551, Japan. Fax: +81-22-237-5215; Tel: +81-22-237-3039; E-mail: matsuura-shunichi@aist.go.jp*

[b] *Taiyo Kagaku Co., Ltd., 1-3 Takaramachi, Yokkaichi, Mie 510-0844, Japan*

Experimental details

Surface modification of TMPS mesopores

As the mesoporous silica capsule for the enzyme, we prepared TMPS10.6 (Taiyo Kagaku Meso Porous Silica, 10.6-nm pore diameter, Fig. S1, Table S1). In order to increase the alkaline tolerance of the framework of the support, the surface of the mesopores of calcined TMPS was treated with a zirconia compound according to the method reported by Yokoyama et al. (Materials Letters, Vol. 65, 67-69, 2011). After the zirconia treatment, the modified TMPS was subsequently treated with 3-glycidoxypropyltrimethoxysilane to prevent leaching of the enzyme from the composite during the enzymatic reaction, as follows: 1 mg of the zirconia-modified TMPS was stirred with 2 mmol of 3-glycidoxypropyltrimethoxysilane in ethanol for over 24 h at 40 °C. The modified TMPS was collected by filtration and then dried for 24 h at 80 °C.

Evaluation of adsorption of glutaminase to TMPS10.6

A batch adsorption experiment was performed by combining 10 mg of each TMPS10.6 powder with 1 mL of 100 mM ethylamine hydrochloride solution (pH 5.5) containing an appropriate amount of glutaminase (0.1–10 mg). The glutaminase–TMPS10.6 mixtures suspended in the solution were gently shaken using a rotator for 20 h at 4 °C. The composites were then centrifuged for 2 min at 14,000 *g* and the supernatant was recovered, after which the enzyme concentration of the first supernatant was determined spectrophotometrically by the bicinchoninic acid (BCA) method to establish the amount of glutaminase adsorbed to the TMPS10.6.

Evaluation of encapsulation of glutaminase in mesopores of TMPS10.6

The encapsulation of glutaminase molecules in the TMPS mesopores was evaluated based on the nitrogen adsorption and desorption measurements of dried powder samples. The measurements were carried out at –196 °C on the BELSORP-max (BEL Japan Inc.) gas adsorption apparatus to evaluate specific surface areas and pore volumes of TMPS10.6 and glutaminase–TMPS10.6 composite. The specific surface areas were calculated by the Brunauer–Emmett–Teller (BET) method using adsorption data ranging from P/P_0 = 0.05 to 0.25.

Preparation of a microreactor containing glutaminase–TMPS10.6 composites

The proposed design of the polydimethylsiloxane (PDMS)/glass microreactor containing glutaminase–mesoporous silica (TMPS10.6) composite particles is shown schematically in Fig. S2. A glass substrate (width: 20 mm, length: 70 mm, thickness: 1 mm; Matsunami Glass Ind., Ltd, Japan) was used as the base material to fabricate the flow channel of the microreactor. To immobilise TMPS10.6, the glass surface of the flow channel (width: 5 mm, length: 50 mm depth: 0.2 mm) fabricated by etching the glass substrate was coated with a thin layer of liquid PDMS prepolymer as the adhesive and then sprinkled with calcined TMPS powder. It was then heated for 3 h at 85 °C. The TMPS particles were then immobilised on the surface by polymerization of a PDMS prepolymer layer (thickness: 0.1 mm); as a result, the final depth of the flow channel was ~0.1 mm (inner volume: ca. 25 μL). The glass surface was then blown with dry air to remove any unattached TMPS, after which the flow channel was sealed with a PDMS membrane (width: 20 mm, length: 70 mm, thickness: 1 mm). The flow channel was fitted with two microtubes for solution supply.

Glutaminase was encapsulated in the pores of the TMPS10.6 immobilised in the microreactor by adding 1 mL of 100 mM ethylamine hydrochloride solution (pH 5.5) containing 0.2 mg mL^{-1} glutaminase into the flow channel at 10 μL min^{-1} at 4 °C using a syringe pump. The obtained glutaminase–TMPS10.6 composites immobilised in the reactor were then rinsed with 2 mL of ethylamine hydrochloride solution at 20 μL min^{-1}.

Enzymatic reactions in batch and microreactor by glutaminase–TMPS10.6 composites

The enzymatic activities of glutaminase–TMPS10.6 composites in the batch experiment were measured for the synthesis reaction forming L-theanine from two substrates, 20 mM glutamine and 100 mM ethylamine (pH 10.2) (Fig. S3). A 1 mL sample of the reaction mixture containing 0.1, 1, and 10 μg glutaminase in the composite form, along with the

substrates, was gently shaken using a rotator for 30 min at 4 °C. The reaction was terminated by adding perchloric acid, after which glutaminase activity was evaluated by determining the amounts of L-theanine using a high-performance liquid chromatograph (Jasco) equipped with an octadecylsilyl-column (Wako). The sample component was separated with the mobile phase (liquid composition; H_2O:methanol:trifluoroacetic acid = 980:20:1) at 0.5 mL min^{-1} for 25 min at 30 °C, and the detection of L-theanine was performed using a UV detector at 210 nm. For comparison purposes, the enzymatic activity of the free enzyme without TMPS10.6 was measured.

In order to assess the flow system, the glutamine–ethylamine reaction solution was added to the flow channel containing the immobilised enzyme. One millilitre of 20 mM glutamine–100 mM ethylamine (pH 10.2) was passed through the reactor at 5, 10, or 20 μL min^{-1}, and the theanine synthesis by the immobilised glutaminase was carried out at various reaction temperatures (4, 30, 50 °C). In cases where the entire microreactor system, including substrate supply, main unit of reactor, and product collection area, were heated (total heating), the temperature in the system was controlled using an air heat-type incubator (MIR-262; SANYO). In cases where the main unit of the reactor within the microreactor system was locally heated (local heating), the temperature in the main reactor was controlled by temperature control units (stage top incubator; Tokai Hit), while both the substrate supply and the product collection area were kept at 4 °C using the air incubator mentioned above. After the reaction, the product solutions were recovered and the glutaminase activity was evaluated by the measuring method previously described.

Evaluation of glutaminase leaching from the glutaminase–TMPS composite in the flow system

In order to evaluate the leaching of glutaminase from glutaminase–TMPS10.6 composite in the flow system, the reaction solution for the theanine synthesis was sequentially added to the flow channel containing the immobilised enzyme. One millilitre of 20 mM glutamine–100 mM ethylamine (pH 10.2) was passed through the reactor at 10 μL min^{-1} at 4 °C for one assay.

To allow determination of the amount of immobilised glutaminase in the microreactor, the enzyme was chemically modified with a thiol-reactive fluorescent dye (Alexa Fluor 488 C_5-maleimide, $C_{30}H_{25}N_4NaO_{12}S_2$; M_r 720.66; $\lambda_{abs}^{max}/\lambda_{em}^{max}$ = 493 nm/516 nm; Invitrogen) at the thiol group position on the enzyme, with a degree of labelling of approximately two dye molecules to one enzyme molecule. Then, the amount of glutaminase immobilised in each

microreactor after the theanine synthesis reaction was directly estimated from standard curves of fluorescence intensities of Alexa 488 dye in the dye-labeled glutaminase–TMPS10.6 composite (0.003~5 μg glutaminase) by a fluorescence-image analyzer (FLA-5100; FUJIFILM).

Figures and Table

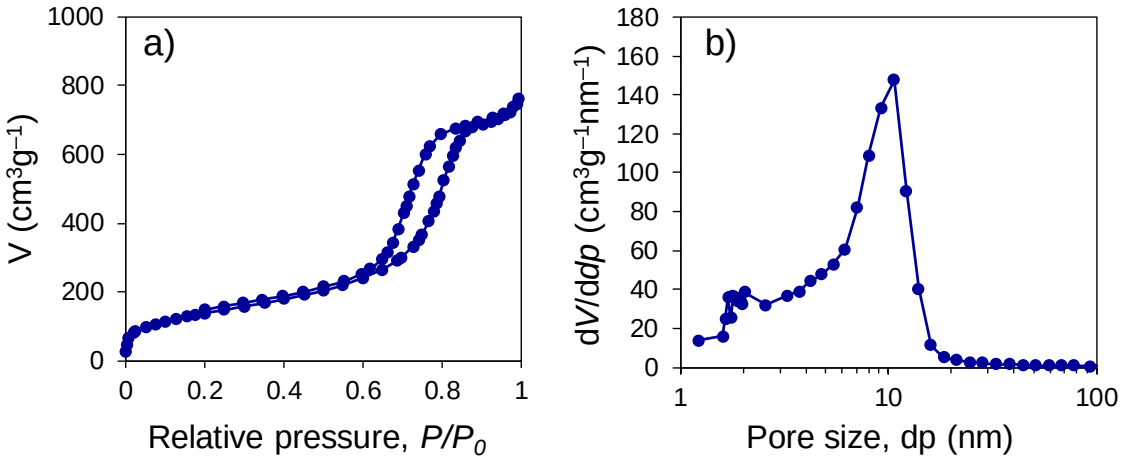

Figure S1. (a) Nitrogen adsorption-desorption isotherm and the corresponding pore size (dp) distribution curve obtained from the adsorption branch by Barret–Joyner–Halenda (BJH) method for TMPS10.6.

Table S1. Physicochemical properties of TMPS10.6.

Pore size (nm)	Specific surface area (m^2g^{-1})	Total pore volume (cm^3g^{-1})
10.6	500.7	1.17

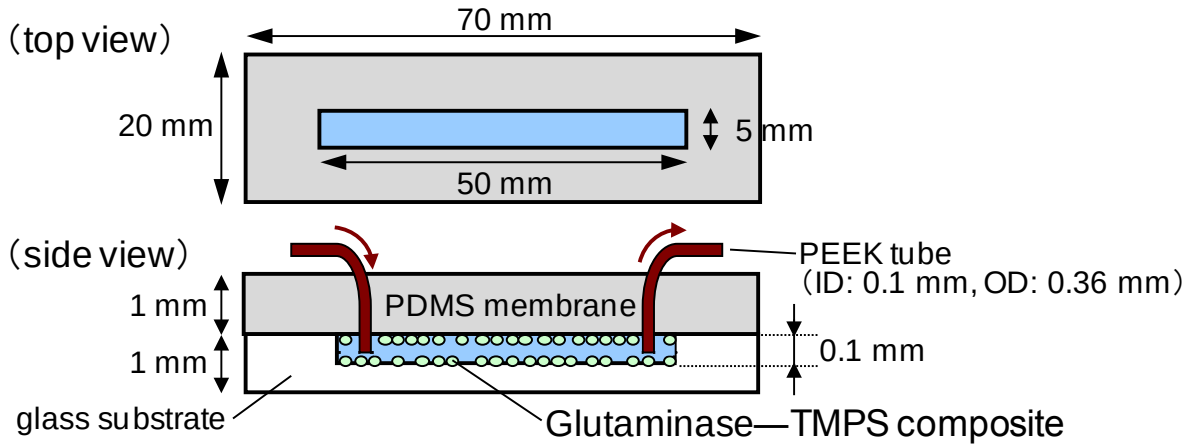

Figure S2. Schematic representation of microreactor with immobilised glutaminase–mesoporous silica (TMPS10.6) composite particles.

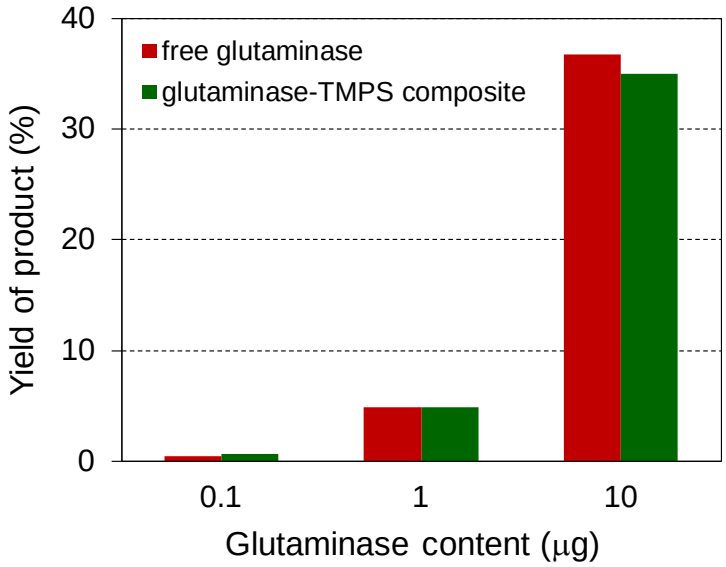

Figure S3. Yield of product (L-theanine) in batch reactions by free glutaminase and glutaminase–TMPS10.6 composite. Reaction conditions: enzyme content, 0.1, 1, and 10 μg; composition of substrate, 20 mM glutamine–100 mM ethylamine (pH 10.2); total volume of substrate, 1 mL; reaction time, 30 min; reaction temperature, 4°C.

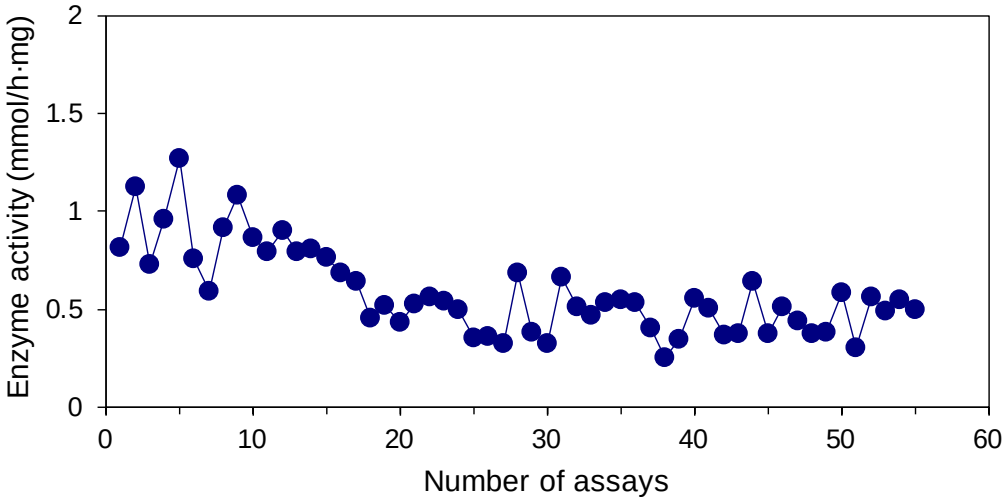

Figure S4. Stability of a microreactor containing glutaminase–TMPS10.6 composites in synthesis of L-theanine. Reaction conditions for one assay: enzyme content, ca. 0.1 μg; composition of substrate, 20 mM glutamine–100 mM ethylamine (pH 10.2); total volume of substrate, 0.3 mL; flow rate of substrate solution, 10 μL min^{-1}; reaction time, 30 min; reaction temperature, 4 °C.

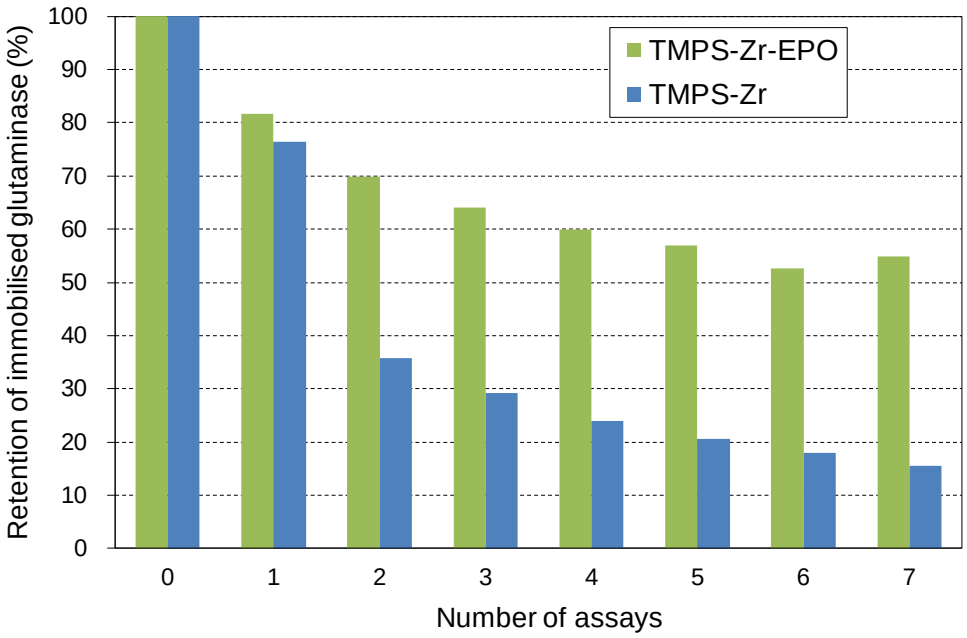

Figure S5. Leaching of glutaminase from glutaminase–TMPS10.6 composite in a repetitive microflow reaction: TMPS-Zr-EPO and TMPS-Zr indicate TMPS modified with a zirconia compound followed by 3-glycidoxypropyltrimethoxysilane and TMPS modified with zirconia compound only, respectively. Reaction conditions for one assay: composition of substrate, 20 mM glutamine–100 mM ethylamine (pH 10.2); total volume of substrate, 1 mL; flow rate of substrate solution, 10 μL min^{-1}; reaction time, 100 min; reaction temperature, 4 °C.