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Preparation of functionalized nanostructures on microchannel surface and their use for enzyme microreactors

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Abstract

Microreactors have become a new and promising technology in chemistry, chemical engineering, and biotechnology fields. Nanostructures have attracted interest as a reaction apparatus especially for catalytic reaction. The present study develops a simple method for nanostructure preparation that is suitably functionalized for a pile-up enzyme microreaction system.

First, we developed a simple method to prepare a nanostructure on a microchannel surface. We applied the sol–gel procedure, which has been utilized for porous structure preparation in batchwise systems. We immobilized the enzyme through amide-bond formation with the surface. The cucumisin-immobilized microreactor showed high efficiency that enable hydrolysis of substrate within 2 s. This result demonstrates that the microreactor has sufficient performance to be capable of small quantity industrial scale processing. Next, we tried to develop a reversible immobilization method for proteins on a microchannel surface. This should solve the enzyme lifetime problem because we are thereby able to detach a denatured enzyme and replace it with a fresh one. We examined two methods, immobilization through disulfide bond and Ni-complex using His-tag. These methods might be useful for further development of highly efficient pile-up enzyme microreaction systems.

Keywords: Microreactors; Sol–gel procedure; Nanostructures

1. Introduction

Miniaturized chemical analysis and synthesis systems have attracted interest recently [1]. Microchannel systems offer several features for chemical reactions including: (1) a microsystem that enables rapid heat exchange which cannot be achieved by a usual batchwise system; (2) rapid mass transfer; (3) a microfluidic system that mainly forms laminar flow; (4) large surface and interface area. Taking advantages of these features, several reaction devices have been reported to demonstrate potential application of such devices. These include highly exothermal reactions, in situ generation of hazardous compounds, efficient solvent extraction, and a rapid energy transfer system. Moreover, many potential applications for miniaturized synthetic reactors are able to use small amounts of catalysts in conjunction with very limited volumes.

Immobilization of catalysts on the surface of insoluble materials has been used as a method allowing reuse of catalysts [2]. In the microreactor system, the microchannel wall surface area is much larger than that of usual batchwise systems. Several catalytic microreactors have been developed, and its advantages have been demonstrated [3,4]. In addition, surface modification methods of microchannel have been developed to increase surface area. Zeolite-immobilized microreactor and porous-silica microchannel devices were applied for catalytic reaction; they yielded higher reaction efficiency [5,6]. However, preparation of these devices is complicated, seemingly unsuitable for routine production of these devices.

Abbreviations: Npys-Cl, 3-nitro-2-pyridinesulfenyl chloride; Suc, succinyl; *p*NA, *p*-nitroanilide; WSCI-HCl, 1-ethyl-3(3-aminopropyl)carbodiimide hydrochloride

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We developed a simple surface modification method using mixtures of 3-aminopropyltriethoxysilane and methyltriethoxysilane in the present study to prepare a porous structure on microchannel surface for enzyme immobilization. We also tried to develop a method to reversibly immobilize proteins to the microchannel surface. This should solve this enzyme lifetime problem because we can detach a denatured enzyme and replace it with a fresh one. We examined two methods: immobilization through disulfide bond and Ni-complex using His-tag.

2. Experimental

2.1. General

Fused silica microcapillary was obtained from GL Science Co. (Tokyo, Japan). Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan) supplied 3-aminopropyltriethoxysilane and methyltriethoxysilane. Other reagents for surface modification were from Wako Pure Chemical Industries Ltd. (Osaka, Japan) or Sigma–Aldrich Co. (St. Louis, MO, USA). Cucumisin was purified by Prof. H. Yonezawa and Dr. K. Arima by a method reported by Kaneda et al. [7]. The lyophilized powder contains 15 units/mg. Water was freshly prepared by Milli-Q (Millipore Corporation, Bedford, MA), and organic solvents were distilled prior to use. The solution was filtered by PTFE filter (0.45 μm) before use, then charged into a 1 ml Hamilton Gastight Syringe (Hamilton Co., Reno, NV, USA). The solutions were supplied to microreactor using a KDS 230 syringe pump with a parallel syringe holder (KD Scientific Inc., New Hope, PA, USA). HPLC analysis was performed by Alliance 2965 system (Waters Corporation, Milford, MA, USA) with a Wakopak C18AR column (3.0 mm × 250 mm) at a flow rate of 0.5 ml/min at 30 °C. Each compound was confirmed with LC–MS (ABI Mariner ESI-TOF system).

2.2. Surface modification

A fused silica microcapillary (320 μm i.d., 20 cm length) was used for microreactor preparation. The microreactor system was assembled as shown in Fig. 1. The capillary was connected to a Teflon tube with a heat-shrinking tube. The Teflon tube was connected to syringe with a PTFE adapter (Flon Chemical Inc., Osaka, Japan). Surface structure was analyzed by scanning electron microscopy (SEM). An FE-SEM image was obtained using a Hitachi S-5200 (Hitachi Co., Tokyo, Japan). A sample was prepared by breaking the microcapillary followed by platinum sputtering on the surface.

2.3. Evaluation of performance of microreactor

The microchannel reactor prepared above was placed in an incubator and maintained at 35 °C. The following substrates were used: Suc–Ala–Ala–Pro–Phe–*p*NA (cucumisin), and pyruvic acid with NADH as a coenzyme (L-lactic dehydrogenase). A freshly prepared and filtered 1 ml solution of substrate (100 μM) in PBS (pH 7.4) was charged using a syringe pump. The resulting solution was collected and analyzed by HPLC to observe changing areas of the substrates and products. HPLC analysis was performed using a Waters Alliance system equipped with Wakosil C-18AR column (3.0 mm × 150 mm) and elution was performed with a linear gradient of 5–50% of acetonitrile in 0.05% TFA over 60 min (for cucumisin) or isocratic elution by 0.05% TFA in water (for lactic dehydrogenase). Each peak was confirmed by MS.

2.4. Batchwise hydrolysis reaction using cucumisin solution

We added 300 μl of 1.3 μM solution of cucumisin to a stirred 2.7 ml solution of Suc–Ala–Ala–Pro–Phe–*p*NA (final

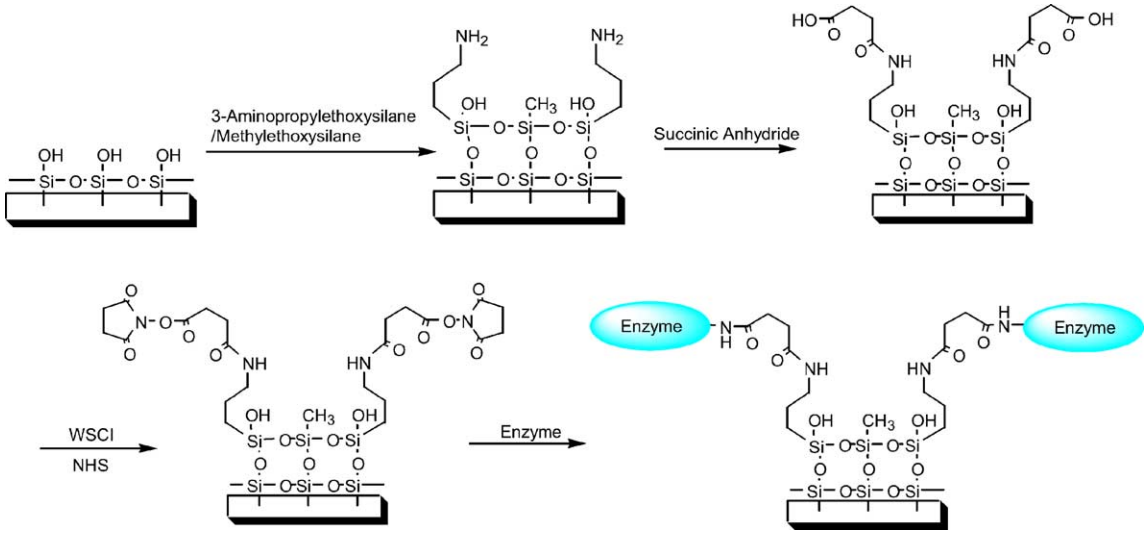

Fig. 1. Surface modification process of microchannel surface.

concentration was 100 μM) in a cuvette. The reaction was monitored by change in optical density at 405 nm with a spectrophotometer (UV-550; Jasco Inc., Tokyo, Japan).

2.5. Batchwise hydrolysis reaction using resin-bound enzymes

NovaSyn TG resin (Novabiochem AG, Switzerland) was swollen in DMF. The resin was modified with succinic anhydride, then the resulting carboxyl group was activated by WSCI–NHS as in a microreactor. This activated resin was reacted with cucumisin for 12 h at 4 °C with constant shaking. After washing with PBS, the amount of immobilized cucumisin was estimated by quantitative amino acid analysis. The immobilized cucumisin (final concentration was controlled to 130 pM) was added to a stirred 10 ml solution of Suc–Ala–Ala–Pro–Phe–*p*NA (final concentration was 100 μM) in a test tube. The reaction was monitored by taking a part of that solution. The solution was filtered and subjected to analysis of absorbance at 405 nm using a spectrophotometer.

3. Results and discussion

3.1. Surface modification of microchannel and its application in microreactor

The present study was designed to develop a simple method to form a nanostructure suitable to immobilize a catalyst on a microchannel surface. We applied the sol–gel procedure, which has been utilized for porous structure preparation in batchwise systems. Fig. 1 shows a typical procedure for surface modification. The microcapillary was treated with piranha solution (7:3 (v/v) mixture of concentrated H_2SO_4:30% H_2O_2) for 12 h at room temperature at a flow rate of 1.0 μl/min, followed by washing with water (total volume was 1 ml). Then the capillary was treated with 3% solution of various mixtures of 3-aminopropyltriethoxysilane and methyltriethoxysilane in 97% ethanol in water for 1 h. After washing with ethanol (total volume was 1 ml), the capillary was heated at 115 °C for 1 h. The number of amino groups immobilized on the microchannel surface was estimated by a modified Gisin's procedure [8] using picric acid; it was determined as 1×10^{17} amino groups/cm^2, meaning that approximately 10 amino groups exist in a 1 Å^2 area. This result suggests that a multilayer structure was formed by silane treatment. Thus, we analyzed the surface image of the microcapillary inner wall. Fig. 2 shows an unusual surface structure which had increased surface roughness, seen on the inner wall; non-treated capillaries had no such structure.

The amino-functionalized microcapillary prepared above was treated with 1 mM solution of succinic anhydride in DMF for 2 h at a flow rate of 5.0 μl/min, to create the carboxyl function. After washing with DMF, the resulting carboxyl group was reacted with 1 M solution of WSCI-HCl and *N*-hydroxysuccinimide in DMF for 1 h, followed by washing with DMF, water and PBS (30 min each). The surface-modified microchannel was filled with a PBS solution of cucumisin (5 mg/ml) and reacted for 12 h at 4 °C without pumping. After washing with PBS, the microcapillary was used for hydrolysis reaction or estimation of immobilized amount of cucumisin. The immobilized enzyme amount was estimated by quantitative amino acid analysis. The cucumisin-immobilized microcapillary was subjected to acid hydrolysis using 6 M HCl. Amino acid analysis was performed by a standard PTC procedure using these hydrolyzed products. The amount of immobilized cucumisin was calculated by comparing with the amino acid composition of cucumisin protein. Thus, we obtained a nanostructured microcapillary by this method and examined its usage as an enzyme microreactor. The number of immobilized enzymes was estimated from quantitative amino acid analysis of hydrolyzed products of this microcapillary. The amount of immobilized enzymes was 130 pmol. Calculating from the size of cucumisin, up to 13 pmol of enzyme molecule can be immobilized by close packing on the microchannel surface as a monolayer. Thus, cucumisin was immobilized as a multilayer; at least 10 layers exist in this microreactor. Moreover, this result supports existence of nanostructures on the microchannel wall.

We examined reaction efficiency using this enzyme-immobilized microreactor. We used hydrolysis of 100 μM phosphate buffered saline solution (pH 7.4) of Suc–Ala–Ala–Pro–Phe–*p*NA for evaluation. The reaction was performed in a thermostatic incubator set to 25 °C. Reaction was completed within 2 s in the microreactor (Fig. 3). According to the flow rate, ca. 4 min was required for 1 ml scale production. In a batchwise system using immobilized enzyme, several hours are required to complete a 1 ml-scale reaction even though the same enzyme/substrate molar ratio as in the inside of microreactor was used and the mixture was vortexed at 1500 rpm (Table 1). Solution-phase batchwise reaction at the same enzyme/substrate molar ratio required more than 10 min to complete at a 1 ml scale. These results demonstrated that the microreactor is an efficient reactor for enzyme reaction. Although the microreactor's small size would seem to preclude industrial scale production, through continuous operation for 1 day, our microreactor can hydrolyze: ca. 360 ml of 100 μM Suc–Ala–Ala–Pro–Phe–*p*NA and can process 22.5 mg of

Table 1
Required time for processing of 1 ml of 100 μM solution of Suc–Ala–Ala–Pro–Phe–*p*NA by bulk and microreaction using same concentration of cucumisin

	Solution (min)	Bead[a] (h)	Microreactor (min)
Required time for complete processing	15	5	1

[a] Cucumisin was immobilized on PEG-PS resin by amide bond.

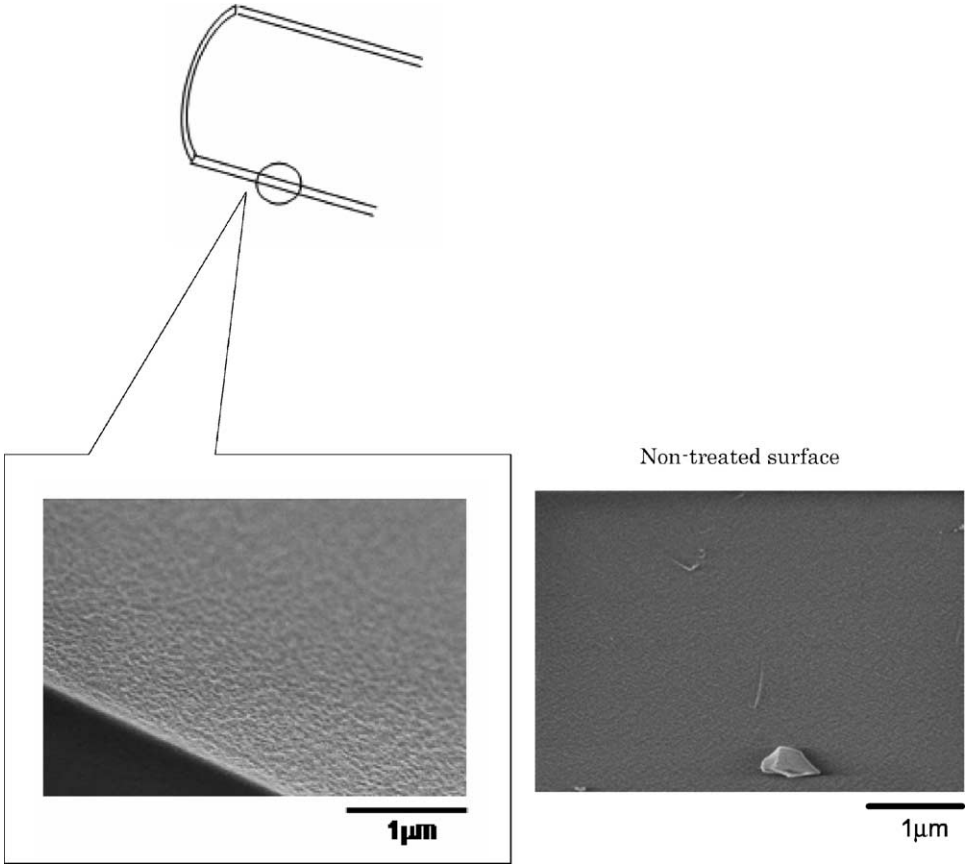

Fig. 2. FE-SEM observation of microchannel surfaces. Surface modified microchannel was broken as shown. The photograph shows edge part of it. Picture of the untreated surface was also shown for comparison.

Suc–Ala–Ala–Pro–Phe–*p*NA with single reactor operation. This amount enables processing of approximately 3 ton per year with 1000 reactors. Therefore, this microreactor is capable of small-batch industrial scale production.

Next, we examined the effect of changing the ratio of silylating reagent on modification level of microchannel surface. This study examines the effect of the ratio of 3-aminopropyltriethoxysilane and methyltriethoxysilane on surface modification. Modification was performed as shown in Fig. 1. First, we observed surface structure using FE-SEM. As in Fig. 2, surface roughness increased in all cases (data not shown). Therefore, the changes in silylating agent ratios did not affect nanostructure formation.

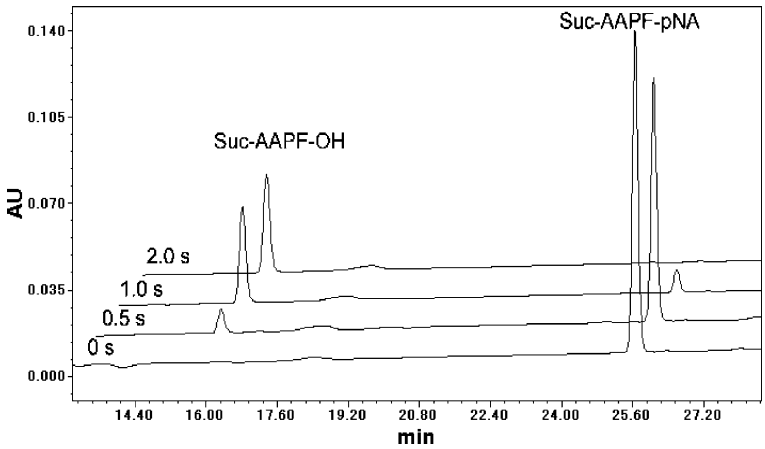

Fig. 3. HPLC chromatogram of hydrolysis product of Suc–Ala–Ala–Pro–Phe–*p*NA by cucumisin-immobilized microreactor. The time given in each chromatogram shows reaction time.

Table 2
Effect of surface modification level on microchannel reactor reaction efficiency

APS[a] (%)	20	40	60	80	100
Amino group per nm^2	375	500	999	951	987
Amount of immobilized cucumisin (pmol)	119	120	120	120	121
Yield[b] (%)	19.1	20.1	20.3	19.9	19.8

[a] Volume percentage of 3-aminopropyltriethoxysilane in total silylating reagent.
[b] Product yield at 0.5 s reaction in microreactor.

We compared the number of immobilized amino groups on the microchannel surface. Table 2 shows those results. In all cases, more than 10 amino groups were created on a 1 nm^2 of the surface. As expected, the number of amino groups increased concomitant with increase of 3-aminopropyltriethoxysilane from 20 to 60%. However, the number of surface amino groups did not increase at 80 and 100% 3-aminopropyltriethoxysilane. These results imply that overly increased content of 3-aminopropyltriethoxysilane does not engender an increased number of surface amino groups. Moreover, we examined the effect of total concentration of silylating agent by increasing total concentration from 3 to 5%, but the number of amino group remained unchanged (data not shown). We prepared cucumisin-immobilized microreactors using the amino-functionalized capillary. Simply loading reagents into the microchannel, as shown in Fig. 1 performed immobilization of the enzyme molecule. First, we examined microreactor performance using capillaries with 400 μm i.d., which was modified with 60% 3-aminopropyltriethoxysilane. With this capillary size, we were able to immobilize ca. 120 pmol of cucumisin. The reaction was accomplished by supplying the substrate solution with syringe pumping. We examined effects of modification level on enzyme immobilization and reactor performance. Table 2 shows that changes in the ratio of 3-aminopropyltriethoxysilane and methyltriethoxysilane did not alter reactor performance or the amount of immobilized enzyme. Thus, changing the ratio of 3-aminopropyltriethoxysilane and methyltriethoxysilane did not alter areas of surface nanostructure of capillaries. Therefore, we were able to immobilize almost identical amounts of enzymes. In addition, too much amino group in the nanostructured area did not affect enzyme immobilization. Perhaps few amino groups were used for immobilization of enzyme. The remainder neither reacted nor interfered with the enzyme reaction.

3.2. Development of reversible immobilization

3.2.1. Immobilization through disulfide bond

Reversible immobilization of proteins to a surface should solve this enzyme lifetime problem because we can detach a denatured enzyme and replace it with a fresh one. The present study is designed to develop a simple method to immobilize an enzyme on a surface reversibly; it has applicability to a microchannel system. We designed the reversible immobilization protocol shown in Fig. 4. The amino-functionalized microcapillary prepared above was treated with a 1 mM solution of succinic anhydride in DMF for 2 h at room temperature at a flow rate of 5.0 μl/min to create carboxyl function. After washing with DMF, the resulting carboxyl group was reacted with a 1 M solution of WSCI-HCl and *N*-hydroxysuccinimide in DMF for 1 h at room temperature, followed by washing with DMF, water and PBS (30 min each). We loaded this microcapillary with a 1 mM PBS solution of cystamine at a flow rate of 5 ml/min for 2.0 h at 25 °C, then washed it with PBS and reduction with 2.4 mM *n*-Bu_3P in 50% MeOH in water for 1.0 h at room temperature. The resulting SH group was activated by reaction with 1 mM solution of Npys-Cl and triethylamine in DMF for 2 h at 25 °C. The resultant SH-activated capillary was washed with DMF and ether, and dried over P_2O_5.

Although Npys-activated thiol groups have been used for reversible affinity labeling, we were not certain whether the system acts in a nanostructured surface environment [9]. Therefore, we examined Npys-modified surface reactivity

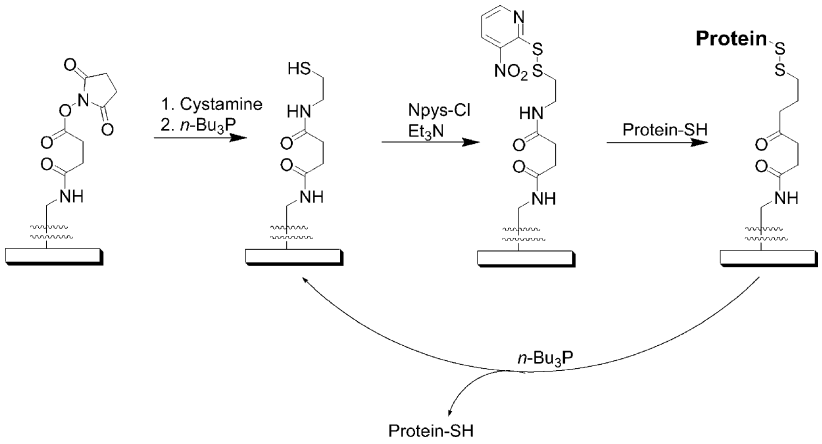

Fig. 4. Reversible immobilization of enzymes through disulfide bond.

with an intact protein molecule. We chose a serine protease cucumisin because it has a free SH group. A PBS solution of cucumisin (50 mg/ml) was immobilized simply by passing it through a microchannel. After washing with PBS, the microchannel was treated with *n*-Bu_3P to remove bound cucumisin from the microchannel surface. The resultant solution was collected and concentrated in vacuo to remove *n*-Bu_3P and methanol. The resultant pellet was dissolved in PBS; the enzyme amount was quantitated using Bradford assay. We immobilized approximately 130 pmol of cucumisin by this method. In our previous study, we were able to immobilize 130 pmol cucumisin by simple amide formation: we were able to immobilize an almost equal amount on the microchannel surface even though the immobilization mode differed. We repeated this immobilization-removal procedure five times, but the immobilized enzyme amount did not change. Therefore, we repeatedly immobilized the same amount of enzyme on a microchannel surface using this method.

Next, we examined whether the enzyme was active or not. We performed a hydrolysis reaction of Suc–Ala–Ala–Pro–Phe–*p*NA. A 100 μM solution of substrate was charged into the microreactor at different flow rates. As shown in Fig. 2, the reaction was completed at a flow rate of 250 μl/min. This result agreed with our previous result using a microreactor which immobilized cucumisin through amide bond. Therefore, performance was maintained with this reactor despite the changed immobilization mode. We repeated immobilization to confirm that removal treatment does not affect microreactor efficiency. We performed immobilization-removal treatment five times and then evaluated substrate hydrolysis activity. Table 3 shows that activity was not altered by repeated immobilization. Therefore, this method is suitable for reversible immobilization of an enzyme in a microchannel. We also compared the lifetime of cucumisin immobilized by this method with a previous one. Disulfide-immobilized cucumisin retained its activity for more than 1 month. This method can be applicable to prepare a small-scale plant because the microreactor prepared this study retained similar performance to the previous one. Moreover, the plant does not require removal and immobilization procedures for more than a month.

3.2.2. Immobilization through His-tag

Although this reversible immobilization method is efficient for immobilization, the method is limited to enzymes containing free SH group on the surface. Therefore, we decided to develop another immobilization method that is widely applicable. His-tag among the proteins was used for purification. We applied this technique for immobilizing a protein on the microchannel surface. We used fused silica capillary for model experiments. The microchannel surface was modified by 1:1 (v/v) mixture of 3-aminopropyltriethoxysilane/methyltriethoxysilane, as usual. The amino group on the surface was modified with succinic anhydride, followed by active ester formation by the WSCI/NHS method. This active ester was reacted with AB-NTA to facilitate formation of a complex with Ni^{2+} ion. The microchannel was ready for immobilization after a solution of nickel sulfate was passed through. The human L-lactic dehydrogenase was used for model study. The cDNA coding L-lactic dehydrogenase was obtained from the cDNA library by PCR, then ligated into pET14b vector, which had been utilized for N-terminal His-tagged proteins. The vector was incorporated into competent cells of BL21(DE3). Using these cells, expression of L-lactic dehydrogenase was performed. We used enzymes purified by Ni-column prepared using Chelating Sepharose FF (Amersham Pharmacia Biotech Inc.). Fig. 5 shows the immobilization and removal steps. The enzyme could be immobilized just passing through the channel with a tethered Ni-complex on the nanostructured surface. Treatment with chelating reagents such as EDTA and nickel sulfate solution can remove the enzyme and regenerate the surface, respectively.

Table 3
Effect of reversible immobilization through disulfide bond for amount of immobilized protein and efficiency of microreactor

	Times				
	1	2	3	4	5
Amount of protein (pmol)	130	129	131	130	130
Reaction yield (%)[a]	19.0	19.5	20.1	20.0	20.1

[a] Product yield of hydrolysis reaction of 100 μM Suc–Ala–Ala–Pro–Phe–*p*NA at 0.5 s.

An amino-functionalized microcapillary prepared as above was treated with 1 mM solution of succinic anhydride in DMF for 2 h at a flow rate of 5.0 μl/min at room temperature to create the carboxyl function. After washing with DMF, the resulting carboxyl group was reacted with 1 M solution of WSCI-HCl and NHS in DMF for 1 h at 25 °C, followed by washing with DMF. A 1 M DMF solution of AB-NTA was loaded to this capillary and reacted for 6 h at a flow rate of 5.0 μl/min at 25 °C. The microcapillary was ready for experiments after several washings with DMF and water.

The entire immobilization procedure was carried out at 4 °C. Enzyme immobilization was performed as follows. The AB-NTA immobilized microcapillary was treated with a 10 mM aqueous solution of nickel sulfate for 12 h at a flow rate of 10 μl/min. After washing with 50 mM Tris–HCl (pH 8.0), the enzyme solution (10 mg/ml) in 50 mM Tris–HCl containing 5 mM of imidazole was loaded into the capillary at a flow rate of 5.0 μl/min. The reaction was continued overnight, then the capillary was washed with 50 mM Tris–HCl containing 5 mM imidazole, then with 50 mM Tris–HCl. Removal of the enzyme was performed using 10 mM solution of EDTA (total volume was 100 μl), followed by several washings with 50 mM Tris–HCl.

The amount of immobilized enzyme was estimated by analyzing the amount of recovered protein by EDTA treatment. Recovered EDTA solution was dialyzed against ammonium

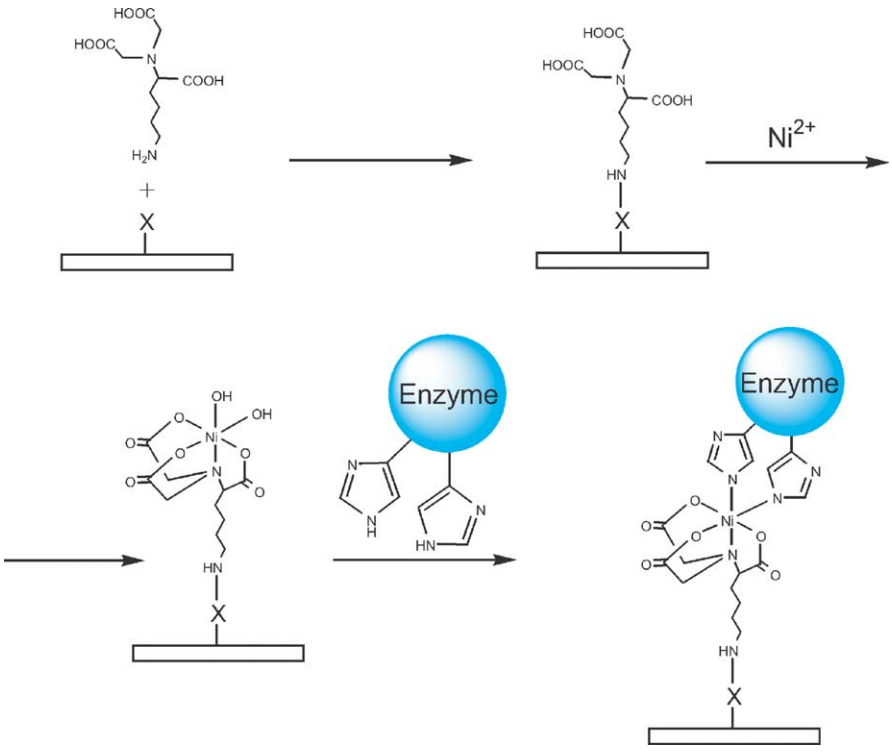

Fig. 5. Reversible immobilization of enzymes through His-tag.

bicarbonate buffer (pH 8.0), and then lyophilized. Resulting powder was dissolved in 100 μl of water, and then analyzed by Bradford assay.

First, we examined validation of this immobilization method. We checked the amount of immobilized enzymes. Table 4 shows that we could immobilize 120 enzyme molecules per 1 nm^2 area, meaning that the enzyme forms in multiple layers on the surface. In addition, this amount was almost the same as the amount of immobilization through amide-bond formation. We repeated the immobilization-removal process for five times and compared the amount of immobilized enzyme. Repetition of immobilization process did not alter the amount of surface enzyme, meaning that reversible immobilization was achieved. We also examined whether the immobilized enzyme is active and the microreactor shows high performance. In most cases, incorporation of His-tag to the terminal of the protein molecule does not alter the protein function. However, most of these results were obtained in solution phase. Therefore, it is not certain that immobilization through His-tag alters protein function. We examined whether immobilization affects the activity of L-lactic dehydrogenase or not. Table 4 shows that the microreactors retained high performance by repeating the immobilization-removal process.

Table 4
Effect of reversible immobilization through His-tag for amount of immobilized protein and efficiency of microreactor

	Times				
	1	2	3	4	5
Amount of protein (pmol)	130	129	131	130	130
Reaction yield[a] (%)	19.0	19.5	20.1	20.0	20.1

[a] Product yield of hydrogenesis of 100 μM pyruvic acid by L-lactic acid dehydrogenase at 0.5 s.

Recent advancement of microreaction technology provides an efficient apparatus for enzyme reactions. We developed a simple method for preparation of nanostructures on a microchannel surface suitable for enzyme microreactors. The reactor showed high performance that expands the possibility of the microreaction system as a small-scale biochemical plant. However, it is difficult to regenerate the reactor upon enzyme denaturation. Our methods overcome this problem. We can remove the denatured enzyme by reduction or simple EDTA treatment and replace it with a new one. This feature might be useful for constructing microchemical plants by increasing their number. Recent progress of genomic analysis enables expansion of the number of applicable enzymes by combination with expression systems. We can develop a microreactor using newly discovered enzymes. Therefore, our method might be useful for future microreaction technology development.

4. Conclusions

The present study developed a simple method to prepare a nanostructure on a microchannel surface. We demonstrated its use in preparing an enzyme microreactor. The result-

ing enzyme-immobilized microreactor showed high performance. We also developed reversible immobilization methods for enzymes on nanostructured microchannel surfaces through disulfide bonding or His-tag. These methods might be useful for development of efficient microchemical devices and plants.

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