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Facile Preparation of an Enzyme-Immobilized Microreactor using a Cross-Linking Enzyme Membrane on a Microchannel Surface

Takeshi Honda,[a] Masaya Miyazaki,[a,b,*] Hiroyuki Nakamura,[a] and Hideaki Maeda[a,b,*]

[a] Nanotechnology Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 807-1 Shuku, Tosu, Saga 841-0052, Japan
Phone: (+81)-942-81-3676, Fax: (+81)-942-81-3657; e-mail: m.miyazaki@aist.go.jp or maeda-h@aist.go.jp
[b] Department of Molecular and Material Science, Interdisciplinary Graduate School of Engineering Sciences, Kyushu University, 6–1 Kasuga Koen, Kasuga, Fukuoka 816-8580, Japan
Phone/Fax: (+81)-92-583-8891

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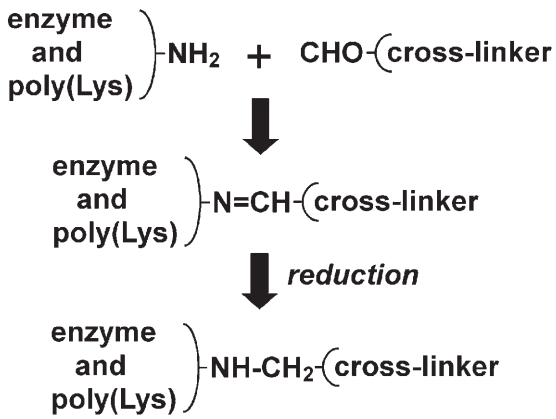
Abstract: The enzyme microreactor has considerable potential for use in biotechnological syntheses and analytical studies. Simplifying the procedure of enzyme immobilization in a microreactor is attractive, and it is achievable by utilizing enzyme immobilization techniques and taking advantage of the characteristics of microfluidics. We previously developed a facile and inexpensive preparation method for an enzyme-immobilized microreactor. The immobilization of enzymes can be achieved by the formation of an enzyme-polymeric membrane on the inner wall of the microchannel through cross-linking polymerization in a laminar flow. However, this method is unsuitable for use in conjunction with electronegative enzymes. Therefore, a novel preparation method using poly-L-lysine [poly(Lys)] as a booster and an adjunct for the effective polymerization of electronegative enzymes was developed in this study. Using aminoacylase as a model for an electronegative enzyme, the reaction conditions for the enzyme-cross-linked aggregation were optimized. On the basis of the determined conditions, an acylase-immobilized tubing microreactor was successfully prepared by cross-linking polymerization in a concentric laminar flow. The resulting microreactor showed a higher stability against heat and organic solvents compared to those of the free enzyme. The developed method using poly(Lys) was applicable to various enzymes with low isoelectric points, suggesting that this microreactor preparation utilizing a cross-linked enzyme in a laminar flow could be expanded to microreactors in which a broad range of functional proteins are employed.

Keywords: cross-linked enzyme aggregation; enzyme microreactor; immobilization; surface modification

Introduction

Microreactors are potentially powerful tools in the fields of chemistry and biotechnology.[1] The excellent performance of microreaction systems is achieved by taking advantage of microchannel system features such as rapid heat and mass transfer, and their larger surface/interface area. These attractive features are favorable for conducting catalytic reactions through catalysts that are immobilized on the reactor.[2] Enzymes are useful catalysts for the production of certain types of chemicals and analysis, which are frequently applied to various reactors. There have been demands for enzymatic microreaction devices including enzyme-immobilized microreactors in several fields, especially for high-throughput biotechnological syntheses and bioanalyses.[3] In such microreactor preparations, nanostructure fabrication and chemical modification of the microchannel are useful technologies.[4] We have also developed a method for immobilizing enzymes on a glass-capillary surface using a chemical modification containing sol-gel techniques.[5] However, these methods require high-level techniques and multi-step procedures, leading to low cost performance. More simple methods using carriers with immobilized enzymes have also been proposed. The carriers were constructed by forming monoliths of a sol-gel and a polymer, and simply incorporating commercial beads and membranes in a microchannel.[6] These structures are often responsible for the



Scheme 1. Reaction of enzyme amino groups and/or poly-(Lys) with aldehyde groups of the cross-linker.

generation of high pressure, which is unfavorable for microsynthesis and -analysis systems with multiple processes based on highly-controlled microfluids,[7] and are energy-intensive to operate. Therefore, in our previous study, we designed an enzyme-immobilized microreactor which was simply and inexpensively prepared and does not require high pressure.[8] In our microreactor process, a substrate membrane covers the internal surface of the microchannel. The cylindrical membrane is composed of the cross-linked polymerized enzyme product. The hollow structure of this reactor prevents high pressure, compared to carrier-filling microreactors.[6,8] In addition, this carrier-free method would be expected to have the advantage of an absence of interactions between the enzyme and the carrier material, which often leads to enzyme inactivation.[9] This bottom-up method is suitable for the formation of miniature structures, and has recently been utilized in various nanotechnological applications such as enzyme-polymerized nanoparticles.[10] The enzyme-polymerized membrane was based on a ***c***ross-***l***inked ***e***nzyme ***a***ggregate (CLEA)[9] formed using a cross-linker with aldehyde groups which react with amino groups of the enzyme (Scheme 1). Therefore, CLEAs could be not obtained for electronegative enzymes due to a lack of reactive amino groups (relatively lower number of lysine residues). In the present study, to demonstrate the applicability of the ***C***LEA-based ***e***nzyme-***m***icroreactor (CEM) method for various enzymes, we developed a novel method for preparing a CEM that is applicable to electronegative enzymes utilizing poly-L-lysine [poly(Lys)]. The successful preparation was found to realize an efficient and fast CLEA formation in electronegative enzymes by using poly(Lys).

Results

The Effect of a Cationic Polymer on CLEA Formation of an Electronegative Protein

The efficient formation of a CLEA is essential for the successful preparation of a CEM. We first explored an efficient method in a batchwise system. Glutaraldehyde (GA) and paraformaldehyde (PA) were used as cross-linkers, as previously described.[8] When mixing the cross-linker and enzyme, CLEAs for electropositive enzymes such as chymotrypsin and trypsin, which contain a high number of lysine residues, are readily formed. On the other hand, no CLEAs were observed in the case of electronegative enzymes. The relatively low content of lysine residues in these enzymes would cause a low degree of cross-linking, leading to inefficient aggregation. To achieve effective cross-linking polymerization, we proposed the close packing of electronegative enzyme molecules by enzyme-trapping using a cationic polymer matrix prior to the cross-linking reaction.[9] The polymer matrix, possessing multiple basic functional groups, and electronegative enzymes will be adsorbed by electrostatic interactions, and form an enzyme-enzyme complex bridged by the matrix. Actually, poly(Lys) immobilized on a carrier is frequently utilized to capture electronegative proteins.[11,12] In this study, we used poly(Lys) as coupling agent for a carrier-free aggregate of the electronegative enzyme.

The effect of poly(Lys) on CLEA formation was compared between chymotrypsin and aminoacylase (hereafter referred to as acylase) as electropositive and electronegative model enzymes, respectively. The turbidity in the reaction mixture increases with increasing amounts of insoluble aggregates. Thus, the progress of CLEA formation could be evaluated by the turbidity of the solution, as estimated by measuring the absorbance at 630 nm. As shown in Figure 1, the cross-linker consisted of GA (0.25%, v/v) and PA (4%, v/v) agglutinated chymotrypsin, but not acylase. No turbidity was observed when concentrated acylase (5-fold; 100 mg mL^{-1}) and/or cross-linker (5-fold; 1.25% GA and 20% PA) was used (data not shown). In contrast, the addition of the cross-linker into the mixture of acylase and poly(Lys) ($M_n = 20$ kDa) led to a remarkable increase in the turbidity of the reaction solution. When mixing acylase and poly(Lys) without cross-linker, in which an acylase-poly(Lys) complex would be formed as a result of electrostatic interactions, the solution contained negligible suspended solids (Figure 1). Poly(Lys) and the cross-linker mixture without acylase, in which the cross-linking reaction would occur between poly(Lys) molecules, showed no turbidity (Figure 1). These results can be attributed to the high hydrophilicity of poly(Lys) which enables each polymeric complex to prevent in-

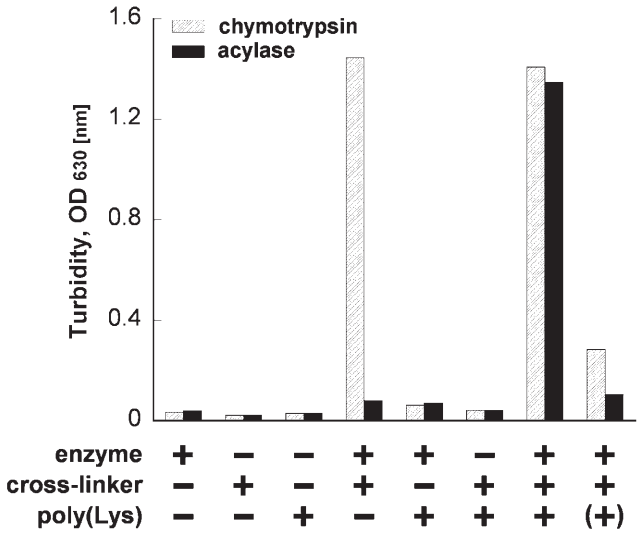


Figure 1. Effect of poly(Lys) on CLEA formation for electropositive chymotrypsin and electronegative acylase. Each bar shows the turbidity of the suspensions after incubation for 60 min at 4 °C. A high turbidity means effective formation of the CLEA. The addition of each solution is described as "+" for each reagent and "−" for buffer without reagents. "(+)" indicates the addition of lysine instead of poly(Lys).

Table 1. CLEA formation in several enzymes of distinct isoelectric points.

Enzyme	p*I*[a]	CLEA[b]
Alkaline phosphatase	5.9	formed with poly(Lys)
L-Aminoacylase	4.0	formed with poly(Lys)
D-Aminoacylase	5.2	formed with poly(Lys)
β-D-Galactosidase	4.6	formed with poly(Lys)
α-Chymotrypsin	8.6	formed without poly(Lys)
Cucumisin	10.4	formed without poly(Lys)
Esterase	5.0	formed with poly(Lys)
Lipase, *Candida rugosa*	4.5	formed with poly(Lys)
Papain	8.8	formed without poly(Lys)
Pepsin A[c]	1.0	formed with poly(Lys)
Subtilisin A	9.4	formed without poly(Lys)
Thermolysin	5.3	formed with poly(Lys)
Trypsin	10.5	formed without poly(Lys)
Tyrosinase	5.2	formed with poly(Lys)
V8 protease	3.7	formed with poly(Lys)

[a] Each value of isoelectric point (p*I*) was determined by isoelectric focusing in previous studies.[31–43] A p*I* value of β-D-galactosidase was obtained from manufacturer's data sheet.

[b] Each CLEA preparation was performed by mixing enzyme (20 mg mL^{-1}) and cross-linker (0.25 % GA and 4 % PA) in the presence or absence of poly(Lys) (M_n = 50 kDa, 10 mg mL^{-1}).

[c] For pepsin, lower pH values (pH 5) was used because pepsin is rapidly and irreversibly denaturated at alkaline values, but is stable until pH 5.5.[44]

solubilization. It is clear that the turbidity of the acylase-poly(Lys)-cross-linker mixture is a result of the concerted reaction of the three reagents. A reaction using lysine instead of poly(Lys) showed no evidence of CLEA formation, indicating the importance of the polymeric structure of poly(Lys) for effective CLEA formation. The addition of lysine to a mixture of chymotrypsin-cross-linker greatly reduced the turbidity of the reaction mixture. This deleterious effect might be due to competition between amino groups of the enzyme and lysine, which possess little cross-linking ability.

In order to achieve broad applicability for CEM for various enzymes, we examined whether CLEAs were formed using poly(Lys), using several industrially useful enzymes with distinct isoelectric points. Table 1 shows the relationship between the isoelectric points of each enzyme and CLEA formation. Electropositive enzymes, including chymotrypsin, cucumisin, papain and trypsin easily formed a CLEA without poly(Lys). In other enzymes, with low isoelectric points (pI < 6), the addition of poly(Lys) permitted successful CLEA formation. These results indicate that poly(Lys) has a strong potential for CLEA formation for a broad range of electronegative enzymes.

Preparation of the Acylase-CEM

Effective CLEA formation in an electronegative enzyme was achieved utilizing poly(Lys). Thus, we applied this technology to a CEM preparation. The electronegative enzyme used was an acylase, which is very interesting from a biotechnology standpoint, because of their relevance in the production of enantiopure amino acids from *N*-acyl derivatives.[13,14] In order to achieve the effective preparation of acylase-CEM with a high enzymatic activity, we optimized the CLEA preparation conditions such as the concentration of cross-linker, the molecular weight of poly(Lys), and the ratio of acylase/poly(Lys) (see Supporting Information, Figures S1 and S2). The acylase-CEM was then prepared at these optimal conditions. The following reagents were used: acylase (20 mg mL^{-1}), poly(Lys) (M_n = 50 kDa, 10 mg mL^{-1}), and cross-linker corresponding to GA (0.03 %) and PA (0.5 %). The acylase solution was mixed with poly(Lys) solution at a 1:1 ratio to form a soluble complex of acylase and poly(Lys). For a simple and inexpensive preparation, a commercially available PTFE tube was employed as a substrate for the CEM (hereafter described as CEM-tube).[8] The CLEA-based membrane in the CEM-tube was formed utilizing a characteristic microflow, namely, a concentric laminar flow consisting of two solutions of the cross-linker and acylase-poly(Lys). This flow was generated by a device consisting of axle dual micropipes, as shown in

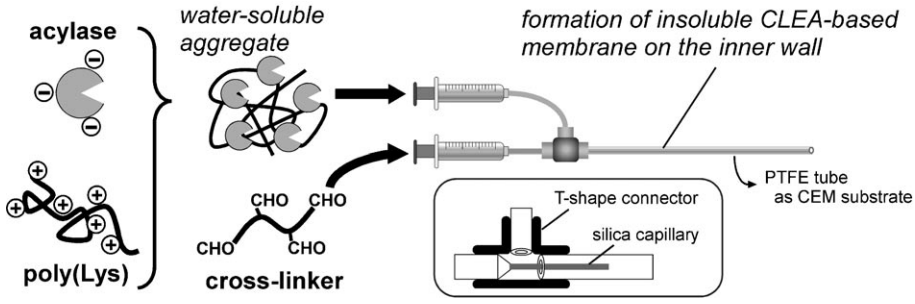


Figure 2. Schematic illustration of the procedure used to prepare an acylase-CEM. The cross-linking polymerization was performed in a concentric laminar flow. A silica capillary was fitted to the outer diameter of the T-shape connector by attaching to a PTFE tube using heat-shrink tubing. The capillary was set in the connector located at the concentric position of the CEM tube. The cross-linker solution was supplied to the substrate PTFE tube through the silica capillary, corresponding to a central stream in the concentric laminar flow. A solution of acylase-poly(Lys) mixture was poured from the other inlet of the T-shape connector, and formed an outer stream of the laminar flow.

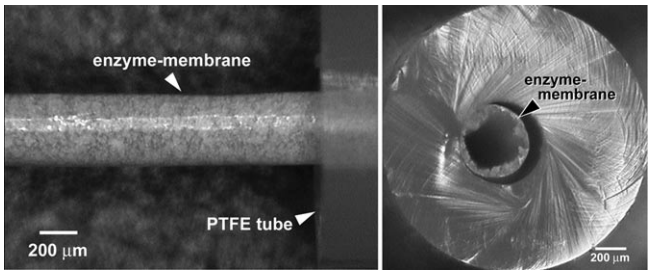


Figure 3. CCD images of a) cylindrical enzyme-membrane (dry state) exposed from the PTFE tube, which forms on the inner wall of the tube and b) a sectional view of the obtained CEM.

Figure 2.[15] A stable laminar flow is important for the successful formation of a CLEA-based membrane on the internal surface of a CEM-tube,[8] and appropriate flow rates should be selected for each solution. We set the pumping rate of the cross-linker (corresponding to a central stream) to a value 12.5 times higher than that of the acylase-poly(Lys) mixture (outer stream). The formation of a concentric laminar flow was confirmed by confocal laser scanning microscopy (see Supporting Information, Figure S3). Calculating from the two distinct pumping rates and microfluidic volumes estimated from the confocal data, average linear velocities of the central and outer streams were 187.6 mm min^{-1} and 6.4 mm min^{-1}, respectively. In the CEM-tube, the residence time of the central stream was much shorter (0.6 min) than that of the outer stream (39.1 min). According to the kinetics of CLEA formation in a batch (see Supporting Information, Figure S4), this residence time for the acylase-poly(Lys) mixture solution was sufficient to form the CLEA.

The resulting CEM was washed with distilled water, dried and the enzyme-membrane observed. Figure 3 shows CCD images of the cylindrical enzyme-membrane (dry state) exposed from the PTFE tube and a sectional view of the CEM. The cylindrical membrane was shrunk by drying, and the thickness of the obtained membrane was 35–60 μm. When a suspension of CLEA prepared in a batchwise system was passed through a PTFE tube, no membrane was observed on the inner wall (data not shown). This result indicates that polymerization in a microfluid enables the enzyme-polymer product to adopt a membrane structure on the inner wall of the CEM tube. The amount of acylase, estimated by a quantitative amino acid analysis as described previously,[8] was 3.25 ± 0.17 nmol per acylase-CEM.

Performance of the Acylase-CEM

The enzymatic performance of the obtained acylase-CEM was evaluated using the hydrolysis of acetyl-D,L-phenylalanine (Ac-D,L-Phe) at various conditions. The CEM was cut off at both ends to a length of 12.2 cm, corresponding to 24 μL of inner volume. The hydrolysis at 4 mM of substrate was complete at this flow rate (1 μL min^{-1}) (Figure 4). The Michaelis–Menten constant (K_m) and maximal velocity (V_{max}), calculated from a Lineweaver–Burke plot, were 4.58 mM and 1.10 mM min^{-1}, respectively. All products showed high enantiopurities (99.3 ± 0.8%) compared to those obtained in a batch process (99.2 ± 0.4%). When treating 100 mM substrate at a flow rate of 4 μL min^{-1}, the highest amount of L-Phe production was 45 nmol per minute. Under similar reaction conditions, the K_m and V_{max} values for the free acylase were estimated to be 2.33 mM and 4.69 mM min^{-1}, respectively. The V_{max} value was somewhat lower than that of the free acylase while a higher K_m value was observed for the immobilized acylase. These results suggest that chemical modification with the cross-linker somewhat suppressed the hydrolytic ability of the acylase.

When a PTFE tube was passed through the acylase-poly(Lys) solution without a cross-linker, no en-

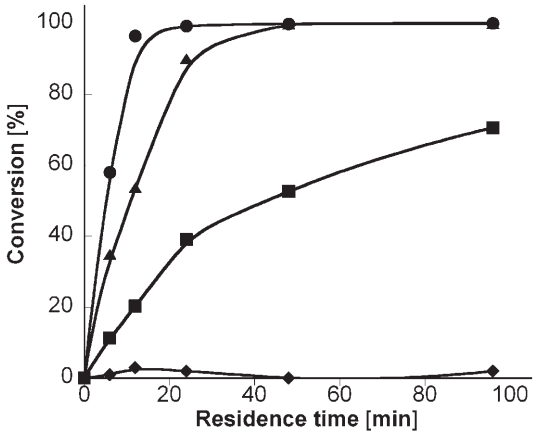


Figure 4. Hydrolysis of Ac-D,L-Phe in the acylase-CEM. A solution of substrate in Tris buffer (pH 8.0) was charged into a CEM tubing (inner volume = 24 μL) for various residence times. The reactions were carried out at three substrate concentrations [4 mM (●), 20 mM (▲), and 100 mM (■)] at 40 °C. This acylase-based reaction converted the L-isomer only. A PTFE tube passed through a mixture of acylase and poly(Lys) had no enzymatic activity (◆).

zymatic activity was observed. This result indicates that the enzymatic activity of the acylase-CEM was due to the acylase immobilized in the CLEA-membrane, and not by non-specific adsorbed free acylase and/or a water soluble acylase-poly(Lys) complex.

It is very important for enzymes incorporated in a microreaction system to have a high stability against conditions such as pH, temperature, and solvent. The pH activity profile of the free acylase and acylase-CEM was obtained by incubating the enzyme in 50 mM phosphate buffer for pH 5–7.5, 50 mM Tris-HCl buffer for pH 8–9 at 40 °C for 30 min. The acylase in the CEM showed a maximum activity at pH 7.0, and was found to be more acid-tolerant than the free acylase (the optimal pH 8.0) (Figure 5a). The thermostability of the enzyme reactor is an important factor regarding its use for industrial applications. To examine the effect of temperature on enzymatic activity, an enzymatic assay was performed after incubating the CEM and free acylase solution in the absence of substrate at various temperatures for 30, 60, and 120 min. The reaction was performed at 40 °C for 30 min. The thermostability of the immobilized acylase in the CEM was superior to that of the free acylase (Figure 5b). In an enzymatic synthesis system, a highly hydrophobic substrate is often unsuitable due to its insolubility. The stability of the enzyme-reactor to organic solvents is very important because organic solvents improve the solubility of certain substrates. Organic solvent tolerances for free acylase and acylase-CEM were estimated using the hydrolytic efficiency in the presence of *N*,*N*-dimethylformamide (DMF). The reaction was performed at 40 °C for 30 min. The acylase-CEM showed a higher resistance to DMF, as compared to the free enzyme (Figure 5c). These stabilizations were observed in α-chymotrypsin-CEM as well.[8]

Discussion

In this study, we describe a novel and facile method for preparing a CEM that is applicable to a broad range of enzymes. The most important factor for the CEM preparation is the efficiency of enzyme-enzyme cross-linking through amino groups. Thus, it is reasonably difficult to prepare a CEM for amino group-poor (electronegative) enzymes. Chemical modification of such an enzyme, for example, by amination using carbodiimide, could facilitate the cross-linking,[16] but it is a rather intricate and time-consuming process. In this study, it was found that the simple addition of a cat-

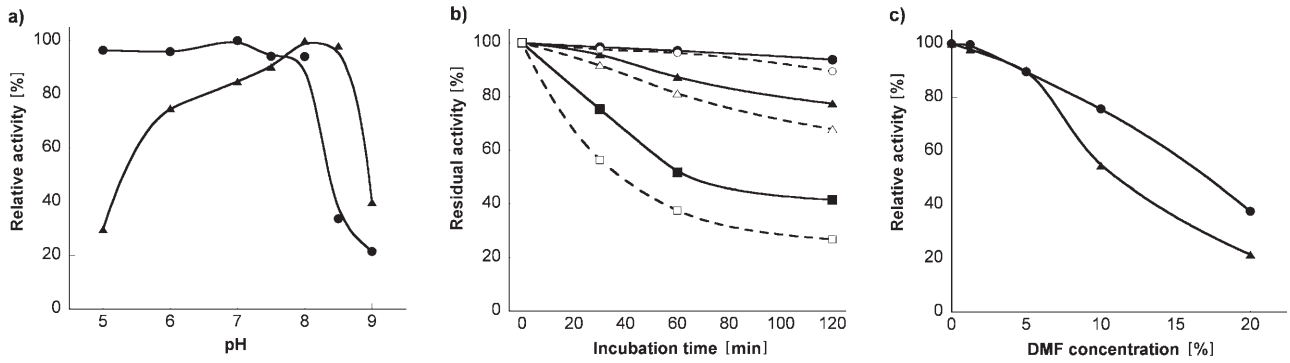


Figure 5. (a) Effect of pH on the enzymatic activity of the acylase-CEM (●) and the free acylase (▲). The activities at the pH optima were assigned values of 100%. (b) Temperature dependence of the enzymatic activity of the acylase-CEM (solid lines) and free acylase (dashed lines) at 40 °C (circles), 50 °C (triangles), and 60 °C (squares). The activities without pre-incubation were assigned values of 100%. (c) Effect of organic solvent on the enzymatic activity of the acylase-CEM (●) and the free acylase (▲). The activities without organic solvent were assigned values of 100%. In all enzyme assays, 4 mM Ac-D,L-Phe was used as substrate.

ionic polymer, poly(Lys), effectively facilitated CLEA formation for an electronegative enzyme (Figure 1, Table 1).

A previous study showed that a cationic polymer could effectively trap an acylase as a result electrostatic interactions.[17] In a similar manner, poly(Lys) would bridge and closely pack (trap) acylase molecules. GA- and PA-conjugated poly(Lys) would also behave as a longer cross-linker possessing more reaction sites per molecule, which would augment the degree of cross-linking as compared to GA and PA. These factors would lead to the effective promotion of an acylase-polymerization based on the covalent cross-linking. A lower-cost method for acylase-CLEA formation has been reported, in which the acylase was precipitated using an inexpensive low molecular precipitant.[18] However, such precipitation (suspension) would be unsuitable for preparing a CEM due to generating unevenness in the membrane and tube obstruction. In contrast, the acylase-poly(Lys) complex showed no precipitation, indicating that poly(Lys) is better suited to the CEM preparation system as a booster of CLEA formation.

When applying this method to various enzymes, the influence on CLEA formation and enzymatic activity should be carefully examined because poly(Lys) often has different characteristics depending on the polymer size.[19–22] For instance, there are interesting reports that the aggregation of an acidic material with poly(Lys) and activation of a protease by poly(Lys) were sensitive to the size of the poly(Lys).[23,24] In our acylase-CLEA, there was little influence of the poly(Lys) size on enzymatic activity. On the other hand, the degree and the kinetics of CLEA formation were dependent on poly(Lys) size (see Supporting Information, Figure S2). These size effects can be attributed to several factors such as electrostatic properties and the number of interaction sites of poly(Lys).

The cross-linker also plays an important role in CEM preparation. It was found that the use of a combination of GA and PA was suitable for acylase-CEM preparation, and that the acylase was more sensitive to the GA/PA than chymotrypsin.[8] For applications of various enzymes to CEM preparations, the total amount of aldehyde reagents and the GA/PA ratio must be optimized for each enzyme.

In this study, a CEM was prepared by utilizing a combination of membrane-formation and microfluidic manipulation. A concentric laminar flow is geometrically appropriate to form the reaction interface along the internal wall of the CEM-tube. In comparison to our previous system, in which a laminar flow consisting of three parallel streams using a simple cross-shaped connector was used,[8] the present system possesses several advantages such as the simplification of pumping work by decreasing the number of inlets, the non-occurrence of aggregates in the inner space of the connector because polymerization began in the CEM-tube (leading to non-obstruction at the connector region), and a more homogeneous and stable formation of the CLEA-based membrane occurred along the inner wall of the CEM-tube.

A previous study reported on a polymer-membrane formation in a microchannel utilizing interfacial polymerization and multilayer flow inside a microchannel.[25] In a similar manner, the enzyme polymerization reaction was considered to mainly occur at the interface between the central and the outer flows, and also at the outer side, in preference to the central stream. When the acylase-poly(Lys) complex diffuses from the outer stream into the central stream and polymerizes at the central region, the complex begins to polymerize and would be unable to have sufficient reaction time (at least 5–10 min from Supporting Information, Figure S4) to mature into an insoluble aggregate due to the shorter residence time (0.6 min) in the central stream. In addition, the diffusion rate of the acylase-poly(Lys) complex from the outer stream to the central stream is lower than the cross-linker from the central to the outer stream. (Roughly calculating based on the literature,[26] the diffusion coefficients are 910 μm^2 sec^{-1} for the cross-linker and 70 μm^2 sec^{-1} for acylase.) These factors would lead to the enzyme-polymer being promoted more preferentially in the vicinity of the inner wall of CEM-tube than at the central region.

It is highly possible that the enzyme of the CEM can have altered enzymatic characteristics compared to the native state. The obtained acylase-CEM showed an decrease in hydrolytic efficiency and an increase in acid-, heat-, and organic solvent-tolerance (Figure 4 and Figure 5). These results were consistent with previous reports on acylase immobilization[11,12,27] and similar results have been observed for other enzymes.[28] Immobilization-based conformational rigidification would allow the acylase to avoid conformational collapse which is responsible for inactivation by heat, pH, and organic solvents. The observed change in pH-sensitivity might also be caused by the modification of amino groups in the acylase and/or the buffering action of poly(Lys) matrix in the microenvironment of the enzyme as described in a previous report.[11]

There were little differences in enzymatic performance between batchwise CLEA and CEM. However, the handling ability of CEM was obviously higher than the batchwise CLEA. For example, in reaction assay and washing procedures, batchwise systems require a series of operations including mechanical agitation, centrifugation, and removal of the supernatant. In the CEM system, such procedures were simply replaced by pumping, leading to more simple, efficient, and accurate handling. Dealing with multiple microscale samples in a batchwise CLEA system is also

considered to be technically difficult and troublesome. The CEM would lead to the establishment of a disposable enzyme-reactor system which enables easy microscale-handling in a multi-parallel manner.

A combination of such advantageous handling ability of CEM and the enzymatic characteristics of acylase has strong potential for the high throughput microprocess of optical separation in a combinatorial enantiopure amino acid production system. Thus, the establishment of such system would be attractive in fields such as basic research of proteins and drug (functional biomaterial) discovery[29] because diverse unnatural amino acids are required on-demand.

In this study, we propose the applicability of the CEM preparation method for other enzymes active in various biotechnological fields as well as acylases. At present, a great interest in such fields is being directed towards the development of combinatorial synthesis and high throughput analysis systems. CEM could have significant potentials as a platform technology of enzyme-microreactions which could be used in the exploration of new applications.

Conclusions

A novel CEM preparation method applicable to electronegative enzymes previously incapable of CLEA formation by our previous method is described. An acylase-CEM model was successfully constructed with an acylase-CLEA using poly(Lys) and cross-linker containing GA and PA under optimal conditions. A cationic bio-polymer poly(Lys) is a useful booster for CLEA formation in the case of electronegative proteins, which closely packs such proteins as a result of electrostatic interactions, and forms a soluble protein-poly(Lys) complex. The simple CLEA preparation method developed here is applicable to various enzymes with distinct isoelectric points, suggesting that this CEM preparation technology could be expanded to a broad range of functional proteins including enzymes. Such broad applicability would lead to the construction of a flexible technology platform for screening and designing a potential protein-immobilized microreactor for broad applications.

Experimental Section

Materials

Poly(tetrafluoroethylene) (PTFE) microtubes (250 μm inner diameter (i.d.), 1.59 mm outer diameter (o.d.) and 500 μm i.d., 1.59 mm o.d.), PTFE adapter, and heat-shrink tubing were obtained from Flon Chemical Inc. (Osaka, Japan). GL Science Co., Ltd. (Tokyo, Japan) provided a silica-fused microcapillary (100 μm i.d., 350 μm o.d.) and a stainless-steel T-shaped connector (Union Tee, SUS-316). When using the microcapillary, the polyimide coating was removed (310 μm o.d.). Glutaraldehyde (GA) and paraformaldehyde (PA) as cross-linker agents, aminoacylase I, D-aminoacylase, α-chymotrypsin, β-D-galactosidase, and other reagents for amino acid analysis were obtained from Wako Pure Chemical Ind., Ltd (Osaka, Japan). Poly-L-lysine hydrobromides of a series of distinct molecular weights were synthesized from N^{ε}-benzyloxycarbonyl-L-lysine *N*-carboxyanhydride as described previously.[30] High molecular weight poly(Lys) (>300 kDa, M_n = 360 kDa), cucumisin, esterase, lipase, papain, pepsin A, thermolysin, sodium cyanoborohydride ($NaCNBH_3$), and other reagents for buffers were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Trypsin, tyrosinase, and V8 protease were purchased from Worthington Biochemical Co., Ltd. (Lakewood, NJ, USA). Alkaline phosphatase was obtained from Biozyme Laboratories Co., Ltd. (San Diego, CA, USA). *N*-Acetyl D,L-phenylalanine (Ac-D,L-Phe), L-phenylalanine (L-Phe), and D-phenylalanine (D-Phe) were purchased from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). All enzymes were ultrafiltered using a microcon YM-10 (molecular weight cut-off = 10 kDa) filter obtained from the Millipore Co. (Bedford, MA, USA). The poly(Lys)s were dialyzed with a dialysis tube (molecular weight cut-off = 500 Da) against phosphate buffer (PB) or gel-filtered in PB for smaller poly(Lys)s.

Typical Preparation of CLEAs in Batchwise System and Measurement of Turbidity

The enzyme and poly(Lys) were dissolved in phosphate-buffered saline; PBS (10 mM, pH 7.2) at final concentrations of 20 and 10 mg mL^{-1}, respectively. The cross-linker reagents containing GA (0.016–0.25 %) and PA (0.25–4 %) in PB (0.1 M, pH 8.0) were used at a constant ratio (1:16, v/v). All solutions were filtered (pore size 0.2 μm). The enzyme, poly(Lys), and cross-linker solutions were mixed at a volume ratio (50 μL:50 μL:100 μL) in a 96-microwell plate (Nunc, Kamstrup, Denmark). After incubation at 4 °C, the turbidity of the mixture was estimated by measuring the absorbance at 630 nm using a microplate reader Multiscan JX (Thermo Electron Co., Milford, MA, USA).

Acylase-CEM Preparation using a Laminar Flow System

The CEM preparation system was assembled as shown in Figure 2. A fused silica microcapillary (5 cm length) and a PTFE tube (250 μm i.d., 5 cm length) for introducing reagents and a PTFE tube (500 μm i.d., 13 cm length) corresponding to the CEM substrate were attached to a T-shaped connector as shown in Figure 2. In order to form a concentric laminar flow in the CEM preparation, the silica capillary was set in the T-shape connector and positioned at the concentric position of the CEM tube. The enzyme and poly(Lys) solutions were mixed at 1:1 volume ratios on ice. All solutions were filtered and charged into 1 mL plastic syringes (Terumo Co., Tokyo, Japan). The cross-linker and enzyme/poly(Lys) solutions were supplied to the CEM tube through the capillary and another inlet of the T-shape connector using a PTFE tube, respectively. Solution introduction was performed at different pumping rates (6.25 μL min^{-1} for the cross-linker and 0.5 μL min^{-1} for the enzyme)

by a Harvard 11 Pico Plus Syringe Pump (Harvard Apparatus, Inc., Holliston, MA, USA). After the polymerization was performed for 3 h, the obtained CEM was rinsed with Tris-HCl (1 M, pH 9.0), which simultaneously quenched any active aldehyde groups remaining on the formed membrane. In order to reduce the resulting Schiff base, CEM was treated with $NaCNBH_3$ in borate buffer (50 mM, pH 9.0). The CEM was extensively washed with PBS (pH 7.5) and no elution of protein was confirmed by measurement of absorbance at 220 nm. All preparations were performed at 4 °C. The obtained CEM was filled with PBS and stored at 4 °C. An enzyme-polymerizing membrane of the obtained CEM was observed using a color CCD digital microscope system (HI-SCOPE DH-2700; Hirox Co., Ltd, Tokyo, Japan).

Estimation of the Amount of Enzyme

To estimate the amount of the immobilized enzyme in the CLEA, a quantitative amino acid analysis was carried out. The tube was lightly washed with distilled water, and the enzyme-membrane was then dried using a vacuum pump, and peeled from the tube by means of a forceps. The obtained membrane was hydrolyzed by HCl (6 M) at 110 °C for 24 h. The CLEA obtained in a batchwise system was also treated in a similar manner. The hydrolyzed product was reacted to phenyl isothiocyanate (PITC), the PITC-labeled amino acids were analyzed on an HPLC system using a Wakosil-PTC (4.0 mm × 200 mm, Wako Pure Chemical Ind., Ltd) with a linear gradient of acetonitrile (60 %, v/v) over 15 min at a flow rate of 1 mL min^{-1} at 25 °C. The protein content was quantified by amino acid analysis using the standard PITC procedure. The amount of the immobilized acylase was calculated by comparison with the amino acid composition of the acylase protein. In the immobilized poly-(Lys), it is very difficult to estimate the precise amount due to the difficulty of detecting cross-linker-conjugated lysine residues by amino acid analysis.

Enzymatic Enantioselective Deacetylation of *N*-Acetyl-D,L-amino Acids

Acylase activity was estimated using a hydrolytic assay with Ac-D,L-Phe as a racemic substrate. The substrate was dissolved in Tris buffer (50 mM), and adjusted the pH 8.0 with NaOH (6 M). In a batchwise system using free acylase and acylase-CLEA, an equal amount of substrate solution was mixed with free acylase dissolved or acylase-CLEA suspended in Tris-HCl buffer (50 mM, pH 8.0) by vigorous stirring at 3,000 rpm in a microtest tube. The enzymatic reaction was terminated by the addition of 0.2 M HCl (final concentration of 0.1 M pH 2.0). In the CEM system, the substrate solution was charged into a 1 mL syringe, and supplied to the tubing CEM using a syringe pump. The flow rate affected the reaction time which corresponded to the residence time in which the substrate solution flowed through the CEM. Standard enzymatic reactions were performed at 40 °C in a thermostated incubator. The reaction product was analyzed by an HPLC system using a Wakosil C18 AR column (3.0 × 250 mm, Wako Pure Chemical Ind., Ltd) with a linear gradient of TFA (0.05 %)-acetonitrile over 30 min at a flow rate of 0.5 mL min^{-1} at 25 °C. The reaction yields were calculated from the peak area calibrated with the standard compound. K_m and V_{max} were computed by fitting the data to the Michaelis–Menten equation using a public domain program, GNUPLOT. The optical yields of the obtained products were calculated from the peaks of L-Phe and D-Phe which were analyzed by HPLC system using a Chirobiotic T column (4.6 × 250 mm, Tokyo Kasei Kogyo Co., Ltd) with an isocratic flow of 50 % ethanol for 12 min at a flow rate of 0.8 mL min^{-1} at 30 °C.

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