Biotechnology and Genetic Engineering Reviews - Vol. 25, 405-428 (2008)

Enzymatic Processing in Microfluidic Reactors

MASAYA MIYAZAKI*[1,2], TAKESHI HONDA[1#], HIROSHI YAMAGUCHI[1], MARIA PORTIA P. BRIONES[1], AND HIDEAKI MAEDA*[1,2,3]

[1]Nanotechnology Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tosu, Saga 841-0052, Japan; [2]Department of Molecular and Material Science, Interdisciplinary Graduate School of Engineering Sciences, Kyushu University, Kasuga, Fukuoka 816-8580, Japan; [3]CREST, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012 , Japan

Abstract

Microreaction technology is an interdisciplinary area of science and engineering. It has attracted the attention of researchers from different fields in the past few years and consequently, several microreactors have been developed. Enzymes are organic catalysts used for the production useful substances in an environmentally friendly way, and have high potential for analytical applications. However, relatively few enzymatic processes have been commercialized because of problems in the stability of enzyme molecule, and the cost and efficiency of the reactions. Thus, there have been demands for innovation in process engineering particularly for enzymatic reactions, and microreaction devices can serve as efficient tools for the development of enzyme processes. In this review, we summarize the recent advances of microchannel reaction technologies and focus our discussion on enzyme microreactors. We discuss the manufacturing process of microreaction devices and the advantages of microreactors compared with the conventional reactors. Fundamental techniques for enzyme microreactors and important applications of this multidisciplinary technology in chemical processing are also included in our topics.

*To whom correspondence may be addressed (m.miyazaki@aist.go.jp or maeda-h@aist.go.jp)
#Present address: Department of Pharmacology, Yamaguchi University Graduate School of Medicine, Yamaguchi 755-8505, Japan

Abbreviations: NTA, *N,N*-bis(carboxymethyl)glycine, which binds Nickel (II) ion, and usually applied for identification/purification of histidine tag of the engineered enzyme molecule; LIGA, Lithography, Electroplating, and Molding; PEEK, poly(ether ether ketone).

Introduction

Microchannel reaction systems, which can be prepared by microfabrication techniques or by assembly and modification of microcapillaries, utilize reaction apparatus of small dimensions (Ehrfeld *et al.*, 2000; Ziaie *et al.*, 2004; Szekeley and Guttman 2005). These systems take advantage of microfluidics or nanofluidics that enables use of micro or nanolitre volumes of reactant solutions, and offer high efficiency performance and repeatability. Therefore, microchannel reaction systems are expected to be the new and promising technology in the fields of Chemistry, Chemical Engineering and Biotechnology (Hessel *et al.*, 2004; Watts and Haswell 2005; Chovan and Guttman 2002; Anderson and van den Berg 2003; Cullen *et al.*, 2004; Wang and Holladay 2005; Miyazaki and Maeda 2006). They offer several advantages for performing chemical reactions over traditional technologies. The key advantages of microsystems include rapid heat exchange and rapid mass transfer that cannot be achieved by a conventional batch system. Unlike macro-scale solution, streams of solutions in a microfluidic system mainly form a laminar flow which allows strict control of reaction conditions and time. In addition, microchannel reaction systems provide large surface and interface areas, which are advantageous for many chemical processes such as extractions and catalytic reactions. Several chemical reaction devices have been reported to demonstrate their potential applications (Hessel *et al.*, 2004; Watts and Haswell 2005; Chovan and Guttman 2002; Anderson and van den Berg 2003; Cullen *et al.*, 2004; Wang and Holladay 2005; Miyazaki and Maeda 2006). Moreover, many potential applications for miniaturized synthetic reactors require only a small volume of catalyst.

Enzymatic conversion has recently come to the forefront because of its environmentally friendly nature. Several enzyme processes have been developed; however, improvement of the entire process is still required to obtain the benefit that can be derived from its use and to gain reevaluation as a common or standard technology (Shoemaker *et al.*, 2003; Garcia-Junceda *et al.*, 2004). Reaction engineering might provide solutions to develop enzyme reaction processes at the commercial level (Schmid *et al.*, 2001), and microreaction engineering is one candidate for such technology. Therefore, several techniques have been developed, either by solution phase or by immobilizing enzymes, to realize enzyme microreaction processes (Miyazaki and Maeda 2006; Urban *et al.*, 2006). In this review, we summarize the recent advances of microchannel reaction technologies especially on enzyme microreactors. We discuss the manufacturing process of microreaction devices and the advantages of microreactors compared with conventional reaction devices. Fundamental techniques underpinning enzyme microreactors and important applications of this multidisciplinary technology are also presented.

Fundamentals of microreactors

For newcomers of the microfluidic reaction techniques, we would like to start our review with a brief introduction. Microfluidic reaction occurs in a small space within the reaction apparatus. Continuous-flow system is mainly employed; in most cases mechanical pumping, commonly by syringe pumping, or electroosmotic flow, which is the motion of ions in a solvent environment through very narrow channels, where an

applied potential across the channels cause the ion migration, are used as the driving force of the reaction system. The microreaction devices that have been developed so far, can be classified into two types, chip-type microreactors and microcapillary devices. Chip-type microreactors offer several advantages including easy control of microfluidics, and integration of many processes into one reaction device. Chip-type microreactors have been mainly used for the development of bioanalytical devices. Manufacturing process of such devices are adaptations mainly from the microelectronics industry. Dry-or wet-etching processes have been used for creating channels on a silicone or glass plates. Polymer-based materials can be used for preparation of enzyme microreactors because most of enzyme reactions have been performed in aqueous solution, especially for bioanalytical use. Polydimethylsiloxane (PDMS), polymethylmarthacrylate (PMMA), polycarbonate, and Teflon have been used for preparation of microreaction devices. These plates could be processed by photolithography, soft lithography, injection molding, embossing, and micromachining with laser or microdrilling. The LIGA (*Lithographie Garbanoforming Abforming*; see Abbreviations) process which consists of a combination of lithography, electrochemical technology and molding, can also be used for the production of microreactors.

In a microreactor, stable formation of laminar streams of different solutions is sometimes required although some cases require better mixing by disrupting laminar streams. Methods for stabilizing multiple laminar flows and micromixing have been developed. Tokeshi *et al* developed guide structures at the bottom of microchannel (Tokeshi *et al.*, 2002). The structures were prepared by wet etching of a glass plate. A laminar stream of organic solvent and water in this microchannel was stabilized by these guide structures. Partial surface modification techniques of microchannels has also been developed. This technique takes advantage of the different surface properties. Organic solvent prefers hydrophobic regions, whereas aqueous solution goes with hydrophilic regions. Modification of glass by octadesylsilane was used to stabilize the flow of organic solvent and aqueous solutions (Hibara *et al.*, 2002). In another study, UV-sensitive self-assembled monolayer with fluorous chain was used for preparing partially-modified microchannel surface (Zhao *et al.*, 2002). Our laboratory developed another method to stabilize solutions (Yamaguchi *et al.*, 2005). Microchannel was fabricated on both bottom and top plates. Microchannel of one of the two plates was coated with gold, then treated with alkanethiol to produce a hydrophobic surface. The resulting microreactor, which forms an upside-down laminar stream, was not only stabilized by interaction with a surface, but also supported by gravity. Overall, such partial modification methods are useful for stabilizing laminar streams under pressure below the critical value. Indeed, the microfluidic phenomenon of laminar flow is one important aspect in the development of chip-type microreactors.

Micromixers which enhance mixing of two or more different solutions in microspace have also been constructed. Rapid mixing in microfluidics is difficult to achieve because under laminar flow mixing of fluids is principally limited to diffusion through the interface. Several micromixers have been developed by adding devices or materials in the microchannel, such as electrokinetical mixing (Erickson and Li 2002) and microbeads (Seong and Crooks 2002). Various types of micromixers which only require structured microchannels have also been developed. These include a chaotic mixer with oriented ridges at the bottom of microchannel (Strook *et al.*, 2002), repeated dividing and merging of fluid (Kim *et al.*, 2002), zig-zag microchannel (Mengeaud *et*

al., 2002), and simple 32 layers in which two solutions are divided into 16 streams and converge to form 32 layers (Yamaguchi 2003). However, the design and fabrication of highly efficient micromixers for effective functioning of microfluidic devices are still desired. The other type of microreaction device consists of a microcapillary. This is the simplest method which does not require any control of microfluidics, rather, it uses a microchannel as the reaction space. The major advantage of this type of microreactor is in scaling up processes which can be achieved by bundling together microcapillaries. Gas or liquid chromatography parts are mainly used to prepare this type of microreactor. Capillary type microreactors are mainly used to develop manufacturing processes, especially catalytic reactions, to take advantage of the large surface area.

Fundamental techniques for enzyme microreactor in solution-phase

CONTINUOUS-FLOW SOLUTION-PHASE REACTION

Simple micro-enzyme reactions have been performed by solution-phase methods. Continuous-flow microreaction has been performed on a chip-type microreactor fabricated from PMMA plate (Miyazaki *et al.*, 2001; Kanno *et al.*, 2002). The reaction was performed by simple loading of substrate and enzyme solutions into separate inlets using syringe pumps. Such reactions rely mainly on rapid mass transfer of the microreaction system. Trypsin-catalyzed hydrolysis of benzoyl-arginine-*p*-nitroanilide (Miyazaki *et al.*, 2001) and glycosidase-catalyzed hydrolysis reactions (Kanno *et al.*, 2002) were performed using this type of microreactor. In all cases, reaction yields were improved 3 to 5 fold in a microchannel system. These results demonstrate the possibility of continuous-flow microreaction systems as a tool for further development of microreaction processes.

Enzyme reaction was also performed in a micromixer format. Glass-bead packed microreaction systems are mainly employed as micromixing devices (Seong *et al.*, 2002, Sotowa *et al.*, 2005). In both cases, efficient reaction has been performed.

STOPPED-FLOW REACTION

Microreactors have been examined not only in the continuous-flow mode but also in the stopped-flow mode of operation. Kitamori and coworkers have described stopped-flow microreactor devices using glass microchips with Y-shaped channels. The stopped-flow procedure involves mobilization of reagents through such a device for a designated period of time using an applied chemical and/or pumped field. The flow is subsequently paused by the removal of the applied field before re-application of the field. Results from experiments utilizing the stopped-flow mode have reported an acceleration of peroxidase-catalyzed reactions (Tanaka *et al.*, 2001)). Such observed increases in the reaction rates using the stopped-flow technique has been attributed to an effective increase in residence time within the device corresponding to the different kinetics associated with these reactions. Stopped-flow microreaction systems with IR heating has also been developed (Tanaka *et al.*, 2000). The system enables non-contact partial heating of the reaction solution. Peroxidase-catalyzed reactions have been performed in a cooled chip equipped with an IR diode laser. The rate of the enzyme reaction

which was initially inhibited due to cooling of the chip to lower the temperature was increased by non-contact heating by utilizing through photothermal effect produced by a diode laser. Their findings suggest the possibility of controlling nanoscale reactions and the precise synthesis of substances by photothermal stimulation.

OTHER SOLUTION-PHASE TECHNIQUES

A multiplex enzyme assay with several simultaneous enzymatic reactions has been performed in an electrophoretic microreaction device (Xue *et al.*, 2001). The resolving power of electrophoresis enables several enzyme assays to be analyzed at high speed. Not only can the activities of individual enzyme catalysts be determined independently of other enzymes but the effects of inhibitors can also be analyzed. This approach enables high throughput analysis on a microchip.

A centrifugal microchip that utilizes a CD player-like apparatus has also been described (Lai *et al.*, 2004). This microreactor does not require the usual pumping system, rather centrifugal and capillary forces are used. This method has been applied to an enzyme-linked immunosorbent assay (ELISA), with each step of the ELISA process carried out by controlling the rotation speed. This method may be useful for the development of analytical microbioreaction systems for multiple analyses of single samples.

Numerical simulation is a strong tool to understand fluid flow and chemical reactions within microchannels. This technique has also been applied to understand the mechanism of enzyme reactions in a microchannel (Sotowa *et al.*, 2008). Such investigations will disclose the mechanism of improved efficiency of enzyme reactions in a microfluidic format.

Fundamental techniques for enzyme immobilized microreactor

ENZYME-IMMOBILIZATION WITHIN MICROCHANNELS

In the development of enzyme processes, the use of immobilized enzymes is preferable. Several methods have been available to immobilize enzymes on supports in conventional reaction apparatus, and these techniques have also been applied to immobilize enzyme within a microspace (*Table 1*).

In batchwise reactors, immobilization of enzymes on beads or monoliths has been used for the separation and recycling of enzymes. This approach has also been applied to microreaction systems. Microreactors with enzyme immobilized on glass beads have been prepared by simply filling the reaction chamber with enzyme-immobilized particles. Such a device was used for the determination of Xanthine using chemiluminescent detection (Richiter *et al.*, 2002). Crooks and co-workers have developed advanced analytical microreactors using enzyme-immobilized microbead-mixing (*Figure 1a*) (Seong and Crooks 2002), and have efficiently performed multistep enzyme reactions using glucose oxidase- and horseradish peroxidase-immobilized polystyrene. In addition, the immobilization of enzyme on Ni-NTA-agarose bead has also been reported. This immobilized enzyme is less denaturated because binding of the enzyme is achieved using a His-tag. This method has been applied to immobilize bacterial P450 (Srinivasan *et al.*, 2004). A similar approach was applied to immobilize

Table 1. Typical techniques for enzyme immobilized microchannel reactor preparation.

Technique	Media	Immobilization method	Immobilized enzyme	Advantage and disadvantage	Ref.
Particle entrapment	Glass	Cross-linking (3-aminopropylsilane/ glutaraldehyde)	• Xantin oxidase • Horseradish peroxidase	• Ease in preparation • Enable multistep reaction • Limited number of enzymes are applicable due to denaturation • Pressure gain	Richiter *et al.* 2002
	Polystylene	Biotin-Avidin (Avidin-coated beads were used)	• Horseradish peroxidase • Glucose oxidase	• Ease in preparation • Enables multistep reaction • Biotin-label is required • Pressure gain	Seong and Crooks 2002
	Agarose	Complex formation (Ni-NTA and His-tag)	• Bacterial P450	• Ease in preparation • Applicable for engineered enzymes • Higher pressure by increasing flow rate and particles may be crushed	Srinivasan *et al.* 2004
	Polystylene	Complex formation (Ni-NTA and His-tag)	• Benzaldehyde liase • p-Nitrobenzyl esterase	• Ease in preparation • Applicable for engineered enzymes • Higher pressure by increasing flow rate and particles may be crushed	Drager *et al.* 2007
	Magnetic bead	Cross-linking (3-aminopropylsilane/ glutaraldehyde)	• Glucose oxidase • Trypsin	• Preparation is easy • Enzyme can be immobilized on any place by placing a magnet • Amount of enzyme particle is limited because of plugging	Nomura *et al.* 2004 Li *et al.* 2007
	Polymer monolith	Entrapment(2-vinyl-4,4-dimethylazlactone, ethylenedimethacrylate, 2-hydroxyethyl methacrylate, acrylamide)	• Trypsin	• Stablization of enzyme structure and activity • Requirement of skill in preparation • Denaturation during entrapment process	Sakai-Kato *et al* 2004

Table 1. Contd.

Technique	Media	Immobilization method	Immobilized enzyme	Advantage and disadvantage	Ref.
	Silica monolith	Entrapment within porous silica	• Trypsin • Protease P	• Stablization of enzyme structure and activity • Compatibility in organic solvent • Requirement of skill in preparation • Denaturation possible during entrapment process	Sakai-Kato*et al*. 2003 Kawakami *et al* 2005, 2007
	Aluminium oxide	Cross-linking (3-aminopropylsilane/ glutaraldehyde)	• Horseradish peroxidase	• Large surface area due to porous nature • Applicable for heterogeneous reactions • Complicated preparation • Not applicable for large-scale processing	Heule *et al*. 2003
Surface modification	SiO_2 surface	Physical adsorption of biotinylated poly-lysine /biotin-avidin	• Alkakine phosphatase	• Ease in preparation • Requirement for avidin- conjugation • Possible occurrence of detachment	Gleason and Carbeck 2004
	PDMS (O_2 Plasma treated)	Physical adsorption of lipid bilayer/biotin-avidin	• Alkakine phosphatase	• Enable immobilization of enzyme on plastic surface • Possible occurrence of detachment • Expensive reagents • Requirement for avidin- conjugation	Mao *et al*. 2002
	PDMS	Physical adsorption of fibrinogen/Photochemmical reaction of Fluorescein- biotin	• Alkaline phosphatase	• Enable partial modification of microchannel • Special equipment is required	Holden *et al* 2004
	Slilicon	Cross-linking (3-aminopropylsilane/ glutaraldehyde)	• Trypsin	• Simple operation • Difficulty in channel preparation • Poor reproducibility	Eckstron *et al*. 2000
	Fused silica (Sol-gel modified)	Cross-linking (3-aminopropylsilane/ succinate)	• Cucumisin • Lipase • L-Lactic dehydrogenase	• Simple operation • Immobilize ~10 times more enzymes than single layer immobilization and therefore, performs with higher reaction efficiency • Several chemistry is available (amide, disulfide, His-tag)	Miyazaki *et al* 2003, 2004, 2005 Kaneno *et al* 2003

Table 1. Contd.

Technique	Media	Immobilization method	Immobilized enzyme	Advantage and disadvantage	Ref.
				• Need several steps for immobilization • Reproducibility strongly affected by characteristics of silica surface	
	PMMA [modified with butyl methacrylate/γ- methyl-acryloxy) propyltrimeth-oxysilicane]	Cross-linking (Si-O bond between modified surface and silica monolyth)	• Trypsin	• Stabilize enzyme under denaturation condition • Complicated preparation method	Qu *et al* 2004
	PDMS (O_2 Plasma treated)	Cross-linking (Si-O-Ti or Si-O-Al bond between titania or alumina monolyth)	• Trypsin	• Stabilizes enzyme under denaturation condition • Complicated preparation method	Wu *et al* 2004
	Fused silica	Cross-linking between physically- immobilized Silica particle (3-aminopropylsilane/ succinate)	• Lipase	• Much larger surface area (1.5 times greater than sol-gel modified surface) and higher efficiency • Complicated preparation method • Unstable withed physical force (bending etc.)	Nakamura *et al* 2004
	Silicon rubber	Cross-linking (3-aminopropyltrieth-oxysilane and glutaraldehyde)	• Thermophilic β-glycosidase	• Reaction can be performed at 80°C • Complicated preparation method • Reaction is slow because not much enzyme can be immobilized	Thomsen *et al*. 2007
	Photopatterning onto PEG-grafted surface	Cross-linking by photo-patterned vinylazlactone	• Horseradish peroxidase • Glucose oxidase	• Reduced non-specific absorption • Sequentially multistep reaction could be achieved • Requires special equipment	Logan *et al*. 2007
	PDMS	Entrapment within hydrogel formed on surface	• Alkaline phosphatase • Urease	• Quite fast reaction (90% conversion at 10 min reaction) • Immobilization of multiple enzyme • Complicated preparation method • Not applicable for higher flow rate	Koh and Psiko 2005

Technique	Media	Immobilization method	Immobilized enzyme	Advantage and disadvantage	Ref.
Membrane	PDMS/Glass	Place PVDF membrane that adsorbs enzymes	• Trypsin	• Easy preparation • Less efficiency • Possibility of leakage at higher flow rate	Gao *et al*. 2001
	Glass	Covalent cross-linking with Nylon membrane formed at liquid-liquid interface (glutaraldehyde)	• Horseradish peroxidase	• Integration of membrane permeation and enzyme reaction • Preparation of multiple membrane • Complicated preparation method • Unstable membrane at higher flow rate	Hisamoto *et al*. 2003
	PTFE	Enzyme-embedded membrane formation using glutaraldehyde/ paraformaldehyde	• α-Chimotrypsin • Trypsin • α-Aminoacylase • Other various enzymes	• Easy preparation • Durable (>40days) • Applicable in organic solvents • Almost all enzymes can be immobilized by adding poly-Lys	Honda *et al*. 2005, 2006, 2007

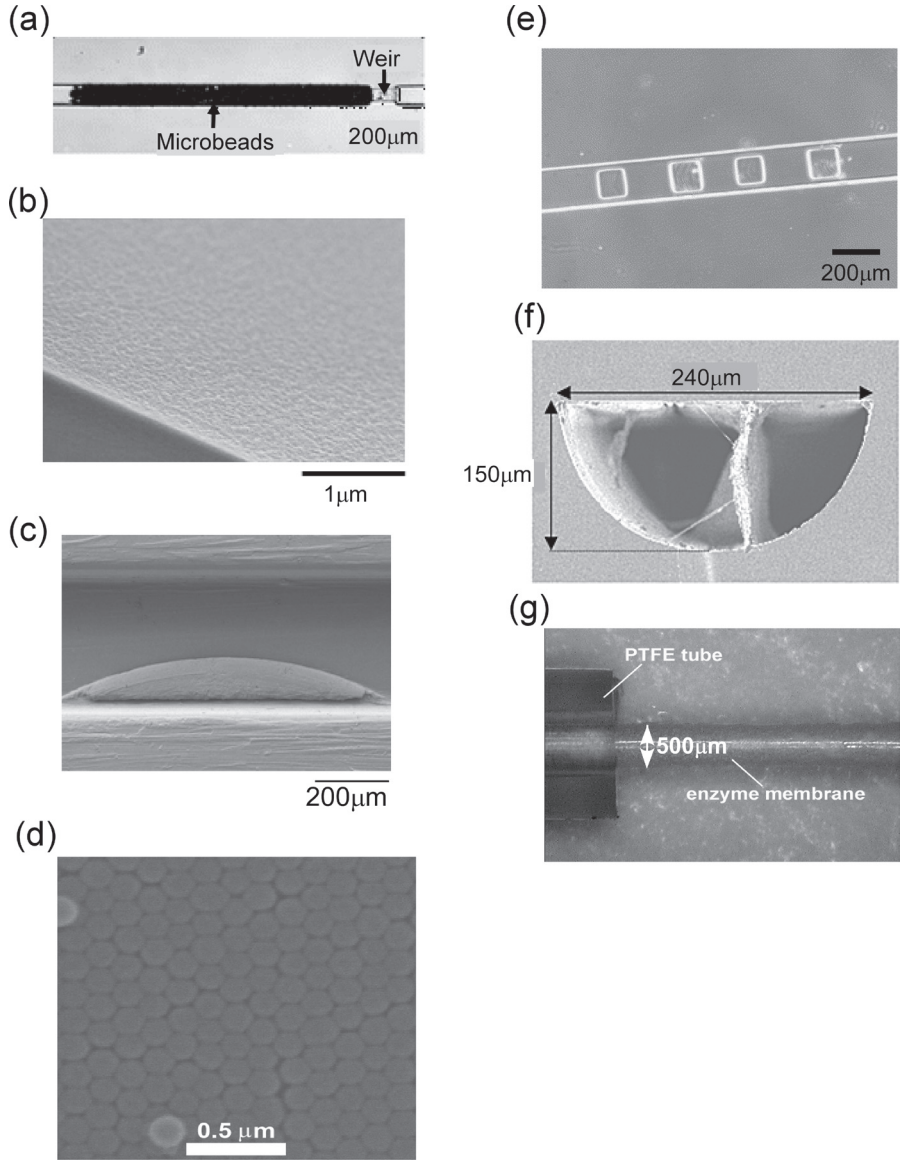


Figure 1. Images of immobilization technique for micro enzyme reactor The enzyme can be easily immobilized by trapping enzyme-immobilized polystylene beads within the microchannel (a). Modified surfaces are also useful for enzyme immobilization. Modified surface obtained by sol-gel technique (b), functionalized microstructure fabricated from silicone rubber (c), nanoparticle arrangement (d), and hydrogel formation (e). Membrane formed within the microchannel can also be used as support for enzyme immobilization. Nylon membrane formed at liquid-liquid interface (f) or membrane of cross-linking enzyme aggregate formed at microchannel surface (g) was used for immobilization. These images were reproduced with permissions from (a) Seong and Crooks (2002), (b) Miyazaki *et al*. (2003), (c) Thomsen *et al*. (2007), (d) Wang *et al.* (2002), (e) Koh and Pishko (2005), (f) Hisamoto *et al.* (2003), (g) Honda *et al*. (2005).

enzymes onto Merrifield resin (Drager *et al.*, 2007). A tyrosine-based Ni-NTA linker was created on the surface of the resin to immobilize His-tagged enzymes. This matrix was loaded into a microstructured channel of the PASSflow™ system. Synthesis of (*R*)-benzoin, (*R*)-2-hydroxy -1-phenylpropan-1-one, and 6-*O*-acetyl-D-glucal were performed using this system. Magnetic beads were also used for enzyme immobilization within the microchannel. Glucose oxidase was immobilized within a Teflon tube with the aid of a magnet (Nomura *et al.*, 2004). The enzyme-immobilized magnet particles were stable and active for more than eight months. This approach was also applied for the preparation of protease-immobilized microreactor for proteomic analysis (Li *et al.*, 2007).

Monolitic microreactors were prepared using several methods. A trypsin-immobilized microreactor was prepared by molding a porous polymer monolith, prepared from 2-vinyl-4,4-dimethylazlactone, ethylene dimethacrylate, and acrylamide or 2-hydroxyethyl methacrylate, with an enzyme, in a microchannel (Sakai-Kato *et al.*, 2004). This microreactor was used for mapping protein digested fragments. Preparation of a microreactor by filling a silica monolith made from tetraethoxysilane with an enzyme and entrapping it within a microchannel has also been developed. Trypsin-encapsulated monolith was fabricated *in situ* on a PMMA microchip to produce an integrated bioreactor that can perform enzymatic digestion, electrophoretic separation and detection in one chip (Sakai-Kato *et al.*, 2003). Another example is a protease-P including monolith prepared from a mixture of tetramethoxysilane and methyltrimethoxysilane (1:4), and that has been used to fill in a PEEK [poly(ether ether ketone)] microcapillary to produce a microreaction system (Kawakami *et al.*, 2005). Aluminum oxide powder can be used as a solid support. Horseradish peroxidase was immobilized on aluminium oxide with 3-aminopropylsilane, and then placed within the microdevice (Heule *et al.*, 2003). This method takes advantage of the porous nature of ceramic microstrut. Overall, preparation of the immobilized enzyme with powdered material or a monolith is significantly easier; however it is unfavorable in large scale processing because of increasing pressure.

IMMOBILIZATION OF ENZYME ON MICROCHANNEL SURFACE

Methods for enzyme immobilization on the microchannel surface have also been developed because they can take advantage of the larger surface area of microreaction systems without gaining pressure. Physical immobilization is an easy way to immobilize molecules. In microchannel systems, a biotin-avidin system has been mainly used to immobilize enzymes. The biotinylated polylysine was physically immobilized onto a glass surface to immobilize streptavidin-conjugated alkakine phosphatase (Gleason and Carbeck 2004). This microreactor was used for rapid determination of enzyme kinetics. A biotinylated lipid bilayer (Mao *et al.*, 2002) and partial biotinylation by photo patterning on fibrinogen (Holden *et al.*, 2004) were also used for immobilization. However, these methods are not suitable for long-term use because of their instability. Also, applications are limited to streptavidin-conjugated enzymes.

The introduction of a functional group on the microchannel surface was used for covalent cross-linking. A trypsin-immobilized microreactor was prepared by modification with 3-aminopropylsilane and glutaraldehyde using the classical method (Eckstrom *et al.*, 2000). Although this immobilization method is easy, fabrication of a

complexed microstructure is required to obtain high performance. Our group developed a modified sol-gel technique to form nanostructures on a silica microchannel surface (*Figure 1b*) (Miyazaki *et al.*, 2003). This method modifies the microchannel surface with polymerized copolymer of 3-aminopropylsilane/ methylsilane. Using this method, an increased surface area has been obtained. Enzymes can be immobilized on these nanostructures by covalent cross-linking through amide-bond formation, disulfide or His-tag, by modifying succinate spacer, at least 10 times more compared with single layer immobilization (Kaneno *et al.*, 2003; Miyazaki *et al.*, 2004; Miyazaki *et al.*, 2005). A microreactor with immobilized cucumisin on the nanostructured surface could process substrate 15 times faster than the batchwise reaction (Miyazaki *et al.*, 2004).

Similar surface modification methods employing sol-gel technique have also been developed (Qu *et al.*, 2004). A PMMA surface was modified with a copolymer of butyl methacrylate/γ- (methylacryloxy)propyltrimethoxysilicane and silica-sol-gel to immobilize enzymes. Using this method, a trypsin-immobilized microreactor was developed. In addition, a trypsin-encapsulated titania and alumina gel matrix was immobilized through the SiOH group formed on a PDMS surface by plasma oxidation (Wu *et al.*, 2004). Using this device, the digestion time was significantly shortened (ca. 2s) and the application for high-throughput protein identification was realized.

Alternatively, silicone rubber material was used for the preparation of functional nanostructure on the microchannel surface (*Figure 1c*: Thomsen *et al.*, 2007). The structure was prepared by micromould fabrication using vinyl-group containing PDMS and silicic acid, and enzyme immobilization by cross-linking with glutalaldehyde. Using this procedure, a microstructured enzyme reactor with immobilized thermophilic β-glycosidasecapable of performing hydrolysis at 80°C was created.

A particle-arrangement technique was also applied to enzyme immobilization. Silica nanoparticles were immobilized onto the surface using a slow evaporation of particle suspension filled-in microchannel (Figure 1d) (Wang *et al.*, 2002). The microchannel obtained was subjected to treatment with 3-aminopropyltriethoxysilane, and immobilization of enzyme was achieved by covalent cross-linking through amino group. Although physical stability needs to be improved, lipase-immobilized microreactor prepared by this method showed 1.5 times faster kinetics than that of microreactor obtained by sol-gel surface modification (Nakamura *et al.*, 2004). This result showed good correlation with the surface area; particle arrangement has approximately 1.5 times larger surface area and could immobilize more enzymes.

Photochemistry was applied to enable selective immobilization of enzymes on the microchannel surface (Logan *et al.*, 2007). In the procedure, vinyl azlaction was photo-grafted onto a PEG-coated polymer surface as a reactive monomer and the enzymes were immobilized through their amino group. This approach was applied for the immobilization of horseradish peroxidase. Another approach for efficient enzyme immobilization is polymer coating. Poly(ethylene glycol)based-hydrogels which incorporate alkaline phosphatase was prepared within a microchannel by exposure to UV light (*Figure 1e*) (Koh and Pishko 2005). This method was also applied to immobilize urease and different enzymes on microchannel surfaces.

MEMBRANE-FORMATION

Enzymes can be immobilized on a membrane within the microchannel. A porous

poly(vinylidene fluoride) membrane embedded within a microchannel can be used for enzyme immobilization. Preparation of a miniaturized membrane reactor by absorption of enzymes onto the membrane has been reported (Gao *et al.*, 2001).

Hisamoto and colleagues have demonstrated that a nylon-membrane formation at the interface of two solutions occurred in a microchannel (*Figure 1f*). Peroxidase was immobilized on this membrane which was used as a chemicofunctional membrane (Hisamoto *et al.*, 2003); however, immobilization of the membrane is technically difficult, and application of this method is limited because the nylon-membrane is unstable in organic solvents.

We have developed a technique that forms an enzyme-immobilizing membrane on the microchannel surface (Honda 2005). This is a modification of CLEA (cross-linked enzyme aggregate) formation, which is used in batchwise organic synthesis (Cao *et al.*, 2006). Simple loading of the enzyme solution and a mixture of glutaraldehyde and paraformaldehyde into the microchannel forms a CLEA membrane on the microchannel wall (*Figure 1g*). The resulting microreactor can be used for prolonged periods (>40 days), and shows excellent stability against organic solvent. Taking these advantages into account, this method is considered ideal for the development of an enzymatic reactor tailored for specific applications. However, this method requires an amino group for immobilization, and application of acidic enzymes with few amino group on their surface is difficult. The application of the approach developed in our laboratory was expanded by adding poly-Lys to aid in membrane formation of acidic proteins (Honda *et al.*, 2006). By this method, almost all enzymes, including highly acidic proteins, can form cross-linked aggregates. We applied this technique to the preparation of an enzyme microreactor, and demonstrated immobilization of several acidic enzymes by this method (Honda *et al.*, 2006). Our results have indicated that almost all enzymes can be immobilized onto the microchannel surface, and that our approach is a robust way of enzyme-immobilized microreactor development.

Advanced applications of micro enzyme reactor for processing

SOLUTION-PHASE ENZYME REACTIONS

Numerous analytical micro-enzyme reactors that take advantage of the reduction of time and the minimal amount of reagents used in microchannel systems (Krenkova *et al.*, 2004) have been developed. However, there have been only a few reports on continuous-flow enzymatic microreaction processes (*Table 2*).

Although the solution-phase reaction is not a favorable process due to the large volume of enzyme required, several important achievements have been reported. Enzymatic oligosaccaride synthesis was performed using β-galactosidase in a continuous-flow microreactor (Kanno *et al.*, 2002a,b). The reaction was performed by separate loading of the enzyme in phosphate buffer solution and the substrate solution in acetonitrile into inlets, and was terminated by heating the recovered solution. The reaction in the microchannel was about 5 times faster than that in the batch reaction.

A biphasic continuous-flow microreaction has also been devised. Goto and co-workers have performed dehalogenation reactions in a chip-type glass microreactor using laccase by separately loading an aqueous solution of enzyme and substrate

Table 2. Enzymatic processing performed in micro enzyme reactor.

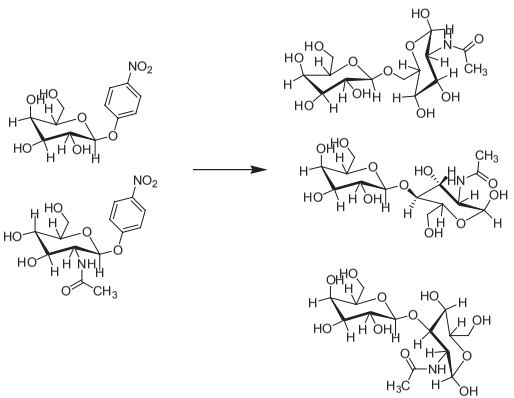
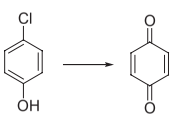
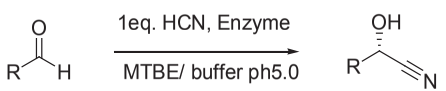
Reaction	Technique	Enzyme	Results	Ref.
	Solution-phase continuous-flow	• ß-Glucosidase	• 5 times better yield was obtained than that of batchwise reaction • Isomers were not isolated	Kanno et al. 2002a, 2002b
	Biphasic solution-phase continuous flow reaction	• Laccase	• Degradation of p-chlorophenol occurred mainly at the aqueous-organic interface in the microchannel • Diffusion of the substrate is the rate-limiting step in the enzymatic degradation	Maruyana et al. 2003
	Plug-flow biphasic system	• Hydroxynitrite lyase	• Asymmetric synthesis was achieved • Product can be separated from enzyme solution and obtained as a MTBE solution.	Koch et al, 2008

Table 2. Contd.

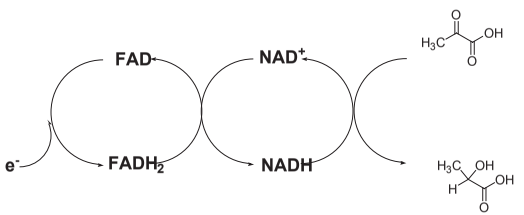
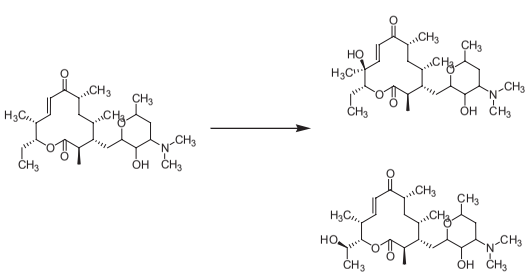
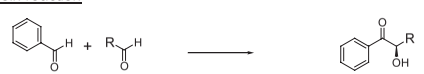
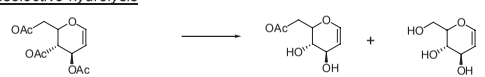
Reaction	Technique	Enzyme	Results	Ref.
	Solution-phase reaction using electrochemical microreactor	• Formate dehydrogenase • Lactate dehydrogenase	• Regenerate coenzymes within single reactor • Regeneration of NADH was as high as 31%	Yoon et al. 2005
	Ni-NTA agarose bead immobilization	• PikC hydroxylase (Bacterial P450)	• >90% conversion was obtained at 70nm/min	Srinivasan et al., 2004
Benzoin reaction  *Regioselective hydrolysis* 	Ni-NTA agarose bead immobilization	• Benzaldehyde liase • p-Nitrobenzyl esterase	• >80% yields were obtained	Drager et al. 2007

Table 2. Contd.

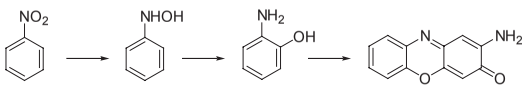
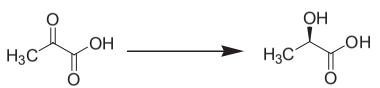
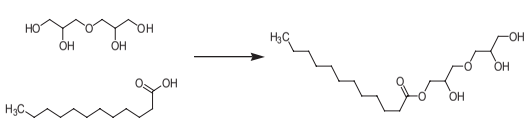
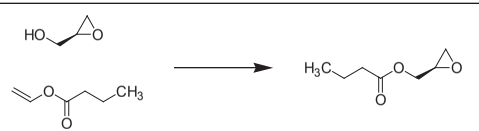
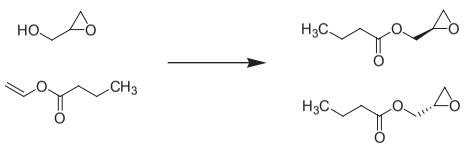
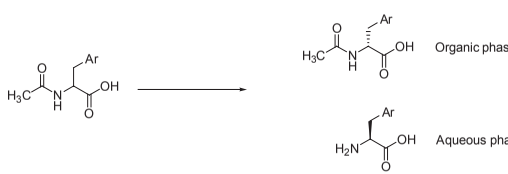
Reaction	Technique	Enzyme	Results	Ref.
	Entrapment of silica-immobilized enzymes within microchannel	• Zinc • Hydroxyaminobenzene mutase • Peroxidase	• Used combinatorial synthesis of 2-aminophenoxyazin-3-one	Luckarift et al. 2007
	Surface modification by sol-gel technique/ Ni-NTA immobilization	• L-Lactic dehydrogenase	• Crude enzyme can be used for immobilization • Reversible immobilization was achieved by EDTA treatment • Reaction was completed within 15 min	Miyazaki et al. 2005
	Surface modification of silica capillary by sol-gel technique/ immobilized through amide bond formation using succinate linker	• Lipase	• 1.5 time better yield was obtained compared with batchwise reaction	Kaneno et al. 2003
	Entrapment of Novozym-435™ within microchannel	• Novozym-435	• Much less of the reactant was required compared with the batchwise test	Garcia et al. 2000

Table 2. Contd.

Reaction	Technique	Enzyme	Results	Ref.
	Silica monolith entrapped within microchannels	• Protease P	• Conversion within microreactor was higher than that of the batchwise reaction at higher flow rates	Kawakami et al. 2005
	Silica monolith entrapped within microchannels	• Lipase	• Optical resolution of products was achieved by connecting commercially available chiral column	Kawakami et al. 2007
	Membrane formation with paraformaldehyde, glutaraldehyde, and poly-Lys	• α-Aminoacylase	• Optical resolution of D/L-amino acids were achieved by connecting to micro solvent extractor	Honda et al. 2007

solution in organic solvent (Maruyama *et al.*, 2003). These researchers performed a detailed kinetic analysis and concluded that the reaction kinetics of a biphasic stream in a microchannel depends on the diffusion of the substrate into the aqueous phase.

A more complicated continuous-flow type enzyme microreaction system was also developed. Regeneration of coenzyme is the most difficult point in an enzyme-catalyzed process. Regeneration of NADH was performed in a Y-shape microreactor, which possesses an electrode within the microchannel (Yoon *et al.*, 2005). Another example has been reported by Koch *et al.* (2008). They used a segment-flow system of methyl-tert-butyl ether and aqueous buffer for asymmetric synthesis of (*S*)-cyanohydrins catalyzed by hydroxynitrile lyase. In their system, clude cell lysate could be used. Although these experiments are primitive, these results demonstrate that a continuous-flow microreactor is one of the more promising devices for the development of efficient enzyme reaction systems.

ENZYME-IMMOBILIZED MICROREACTOR FOR PROCESSING

Applications of enzyme-immobilized microreactors for processing have also been presented including hydroxylation of macrolide in a microreactor (Srinivasan *et al.*, 2004). PikC hydroxylase was immobilized on Ni-NTA agarose beads, and then filled into the microchannel. This microreactor was used for hydroxylation to produce methymycin and neomethylmycin, and over 90% conversion was achieved at a flow rate of 70nl/min. Such high efficiency might have resulted from shorter residence times, which is preferable for enzymes with inherent stability. A similar immobilization technique was applied for the synthesis of (*R*)-benzoin, (*R*)-2-hydroxy-1-phenylpropan-1-one, and 6-*O*-acetyl-D-glucal (Drager *et al.* 2007). His-tagged protein was directly immobilized within the microstructured PASS*flow* reaction system through tyrosine-based Ni-NTA system.

The application of enzyme-immobilized microreactor for multistep synthesis has also been demonstrated (Luckarift *et al.*, 2007). Three separated microfluidic devices, which possess metallic zinc, silica-immobilized hydroxyaminobenzene mutase, and silica-immobilized peroxidase within a microchannel, were prepared and connected sequentially. These devices were used for combinatorial synthesis of 2-aminophenoxyazin-3-one. These results open the door for the application of micro bioreactors for enzymatic synthesis of bioactive natural products.

Esterification and hydrolysis reactions are important processes in industry that have also been performed in a microchannel system. Lipase-immobilized microreactors have been prepared using a ceramic microreactor and glass microcapillary (Kaneno *et al.*, 2003), wherein hydrolysis of the ester was conducted**.** Both microreactors showed 1.5 times better yield than the batchwise reaction using the same volume/enzyme ratios. This could have resulted from an increase in contact due to the larger surface area of microchannel systems. A microreaction using immobilized Novozym-435™ was also reported, where esterification of diglycerol with lauric acid was performed (Garcia *et al.*, 2000). A monolytic microreactor tethering protease P was applied for bioconversion process. Transesterification of (*S*)-(-)-glycidol and vinyl *n*-butyrate was performed using this microreaction device (Kawakami *et al.*, 2005) but the conversion depended on the amount of immobilized enzyme. Similary, they separated a racemic product

which was obtained by reaction in a lipase-entrapped microreactor, by connecting chiral column sequentially to the microreactor (Kawakami *et al.*, 2007).

We have developed a novel integrated microreaction system which combined an enzyme microreactor with a solvent extractor. The enzyme-immobilized microreactor was prepared by membrane-formation technique using α-aminoacylase with poly-Lys (Honda *et al.*, 2006). This microreactor was connected with a microextractor which has a partially modified microchannel (Yamaguchi *et al.*, 2005). Using this microreaction system, optical resolution of D/L-phenylalanine analogs was performed. The D-phenylalanine analogs were obtained efficiently with high optical purity (Honda *et al.*, 2007).

So far, few enzymes have been applied for microreaction process development.

Concluding remarks

Microchannel devices can be useful in imitating various biological reaction apparatus, such as the cellular surface and vascular system, by providing the advantages of limited space and laminar flow compared with the conventional reaction apparatus. The quest for microreaction technologies will lead to better process intensification and efficient analytical methods. Increasingly, new findings are being achieved in microfluidics. Further investigation on microfluidics could provide novel mechanisms not observed in conventional systems, and better understanding of fluidics in microchannels might enable new reaction pathways not possible with the conventional systems.

The strong advantages offered by microreaction devices are useful, particularly in the development of microreaction systems for commercial purposes. Once a microreactor has been optimized, it can be easily introduced into an industrial-scale plant. Parallel scale-out enables extension of reaction conditions optimized in a single reactor, and eliminates scale-up problems arising from conventional processes. Parallel operation of the same microreaction provides high throughput operation of different reagents at a single operation and serves as an excellent tool for combinatorial processing. Although several problems, such as connection, parallel control of fluid and reaction conditions, and monitoring, are common challenges, the benefits offered by microreaction technology accelerate the development of enzyme reaction devices.

As described here, few enzymes have been applied to microreaction process development, and not so many patents describing the construction of micro enzyme reactors have been thusfar published. This is a clear indication that the field is still in its initial stage. Efforts directed to the development, optimization and application of micro enzyme reactors will open a new era for biochemical processing.

Acknowledgments

We thank Professors Shigeharu Morooka (Fukuoka University), (Fukuoka Women's University), Masayuki Fujii (Kinki University), Kazunari Arima (Kagoshima University) and Kenichi Kanno (Kinki University) for their support. The technical assistance of Jun Kaneno (NS Materials Co.) and other colleagues in the laboratory are also acknowledged. Part of this work was supported by grants-in-aid for scientific research (B) (20310074) from Japan Society for the Promotion of Science.

References

Anderson, H. and Van Den Berg, A. (2003) Microfluidic devices for cellomics: a review. *Senor and. Actuators B,* **92**, 315-325.

Cao, L., Van Langen, L., and Sheldon, R. A. (2003) Immobilized enzymes: carrier-bound or carrier-free? *Current Opinion in Biotechnology*, **14**, 387-394

Chovan T. and Guttman, A. (2002) Microfabricated devices in biotechnology and biochemical processing. *Trends in Biotechnology.*, **20**, 116-122

Cullen, C. J., Wootton, R. C. R., and De Mello, A. J. (2004) Microfluidic systems for high-throughput and combinatorial chemistry *Current Opinion in Drug Discoverly & Development*, **7**, 798-806.

Drager, G., Kiss, C., Kunz, U., and Kirschning, A. (2007) Enzyme-purification and catalytic transformations in a microstructured PASS*flow* reactor using a new tyrosine-based Ni-NTA linker system attached to a polyvinylpyrrolidinone-based matrix. *Organic and Biomolecular Chemistry*, **5**, 3657-3664.

Ekstrom, S., Onnerfjord, P., Nilsson, J., Bengtsson, M., Laurell, T., and Marko-Varga, G. (2000) Integrated microanalytical technology enabling rapid and automated protein identification. *Analytical Chemistry*, **72**, 286-293

Ehrfeld, W., Hessel, V., and Lowe, H. (2000) *Microreactors-New Technology for Modern Chemistry*, Weinheim, Wiley-VCH.

Erickson, D. and Li, D. (2002) Influence of surface heterogeneity on electrokinetically driven microfluidic mixing. *Langmuir* **18**, 1883-1892

Gao, J., Xu, J., Locascio, L.E., and Lee, C. S. (2001) Integrated microfluidic system enabling protein digestion, peptide separation, and protein identification. *Analytical Chemistry,* **73**, 2648-2655

Garcia-Junceda, E, Garcia-Garcia, J. F., Bastida, A., and Fernandez-Mayoralas, A. (2004) Enzymes in the synthesis of bioactive compounds: the prodigious decades. *Bioorganic and Medicinal Chemistry*, **12**, 1817-1834

Garcia,, E., Ferrari, F., Garcia, T., Martinez, M., and Aracil, J. (2000) Use of microreactors in biotransformation processes: study of the synthesis of diglycerol monolaurate ester. in *Proceedings of the 4th International Conference on Microreaction Technology (IMRET 4)*, 5-9, New York, American Institute of Chemical Engineers.

Gleason, N. J. and Carbeck, J. D. (2004) Measurement of enzyme kinetics using microscaler steady-state kinetic analysis. *Langmuir*, **20**, 6374-6381

Hessel, V., Hardt S. and Lowe, H. (2004) *Chemical Micro Process Engineering*, Weinheim, Wiley-VCH.

Heule, M., Rezwan, K., Cavalli, L., and Gauckler, L. J. (2003) A miniaturized enzyme reactor based on hierarchically shaped porous ceramic microstruts. *Advanced Materials*, **15**, 1191-1194.

Hibara, A., Nonaka, M., Hisamoto, H., Uchiyama, K., Kikutani, Y., Tokeshi, M, and Kitamori, T. (2002) Stabilization of liquid interface and control of two-phase confluence and separation in glass microchips by utilizing octadecylsilane modification of microchannels. *Analytical Chemistry*, **74**, 1724-1728

Hisamoto, H., Shimizu, Y., Uchiyama, K., Tokeshi, M., Kikutani, Y., Hibara, A., Kitamori, T. (2003) Chemico-functional membrane for integrated chemical processes on a microchip. *Analytical Chemistry,* 75, 350-354.

Holden, M. A., Jung, S.-Y., and Cremer, P. S. (2004) Patterning enzymes inside microfluidic channels via photoattachment chemistry. *Analytical Chemistry*, **76**, 1838-1843.

Honda, T., Miyazaki, M., Nakamura, H., and Maeda, H. (2005) Immobilization of enzymes on microchannel surface through cross-linking polymerization. *Chemical Commun*ications, 5062-5064

Honda, T., Miyazaki, M., Nakamura, H., and Maeda, H. (2006) Facile preparation of an enzyme-immobilized microreactor using a cross-linking enzyme membrane on a microchannel surface. *Advanced Synthesis & Catalysis*, **348**, 2163-2171.

Honda, T., Miyazaki, M., Yamaguchi, Y., Nakamura, H., and Maeda, H. (2007) Integrated microreaction system for optical resolution of racemic amino acids. *Lab on a Chip*, **7**, 366-372 2007

Kaneno, J., Kohama, R., Miyazaki, M., Uehara, M., Kanno, K., Fujii, M., Shimizu, H., and Maeda, H. (2003) A simple method for surface modification of microchannels. *New Journal of Chemistry*, **27**, 1765-1768

Kanno, K., Maeda, H., Izumo, S., Ikuno, M., Takeshita, K., Tashiro, A., and Fujii, M. (2002a) Rapid enzymatic transglycosylation and oligosaccharide synthesis in a microchip reactor. *Lab on a Chip* **2**, 15-18

Kanno, K., Kawazumi, H., Miyazaki, M., Maeda, H., and Fujii, M. (2002b) Enhanced enzymatic reactions in a microchannel reactor. *Australian Journal of Chemistry*, **55**, 687-690.

Koch, K., Van Den Berg, R. J. F., Nieuwland, P. J., Wijtmans, R., Wubbolts, M. G., Shoemaker, H. E., Rutjes, F. P. J. T., and Van Hest, J. C. M. (2008) Enzymatic synthesis of optically pure cyanohydrins in microchannel using a crude lysate. *Chem. Eng. J.*, **135S**, S89-S92.

Kawakami, K., Sera, Y., Sakai, S., Ono, T., and Ijima, H. (2005) Development and characterization of a silica monolith immobilized enzyme micro-bioreactor. *Industrial and Engineering Chemical Research*, **44**, 236-240

Kawakami, K., Abe, D., Urakawa, T., Kawashima, A., Oda, Y., Takahashi, R., and Sakai, S. (2007) Development of a silica monolith microbioreactor entrapping highly activated lipase and an experiment toward integration with chromatographic separation of chiral esters. *Journal of Separation Science*, **30**, 3077-3084.

Kim, J.-H., Kin, B.-G., La, M., Yoon, J.-B., and Yoon, E. (2002) A disposable passive microfluidic system integrated with micromixer and DNA purification chip for DNA sample preparation. in *Micro Total Analysis System 2002*, eds Y. Baba, S. Shoji and A. van den Berg, Vol. 1, 224-226, Dordrecht: Kluwer Academic Publishers.

Koh, W.-G. and Pishko, M. (2005) Immobilization of multi-enzyme microreactors inside microfluidic devices. *Sensor and Actuators B*, **106**, 335-342.

Krenkova, J. and Foret, F. (2004) Immobilized microfluidic enzymatic reactors. *Electrophoresis*, **25**, 3550-3563

Lai, S., Wang, S. N., Luo, J., Lee, L. J., Yang, S. T., and Madou, M. J. (2004) Design of a compact disk-like microfluidic platform for enzyme-linked immunosorbent assay. *Analytical Chem*istry, **76**, 1832-1837.

Li, Y., Xu, X., Yan, B., Deng, C., Yu, W., Yang, P., and Zhang, X. (2007) Microchip reactor packed with metal-ion chelated magnetic silica microspheres for highly efficient proteolysis. *Journal of Proteome Research*, **6**, 2367-2375.

Logan, T. C., Clark, D. S., Stachowiak, T. B., Svec, F., and Frechet, J. M. J. (2007)

Photopatterning enzymes on polymer monoliths in microfluidic devices for steady-state kinetic analysis and spatially separated multi-enzyme reactions. *Analytical Chemistry*, **79**, 6592-6598.

Luckarift, H. R., Ku, B. S., Dordick, J. S., and Spain, J. C. (2007) Silica-immobilized enzymes for multi-step synthesis in microfluidic devices. *Biotechnology and Bioengineering*, **98**, 701-705.

Maruyama, T., Uchida, J., Ohkawa, T., Futami, T., Katayama, K., Niishizawa, K., Sotowa, K., Kubota, F., Kamiya, N., and Goto, M. (2003) Enzymatic degradation of *p*-chlorophenol in a two-phase flow microchannel system. *Lab on a Chip* **3**, 308-312

Mengeaud, V., Josserland, J., and Girault, H. H. (2002) Mixing processes in a zigzag microchannel: Finite element simulations and optical study. *Analytical Chemistry*, 74, 4279 -4286

Miyazaki, M., Nakamura, H., and Maeda, H. (2001) Improved yield of enzyme reaction in microchannel reactor. *Chemistry Letters*, 442-443

Miyazaki, M., Kaneno, J., Uehara, M., Fujii, M., Shimizu, H., and Maeda, H. (2003) Simple method for preparation of nanostructure on microchannel surface and its usage for enzyme-immobilization. *Chemical Communications*, 648-649

Miyazaki, M., Kaneno, J., Kohama, R., Uehara, M., Kanno, K., Fujii, M., Shimizu, H., and Maeda, H. (2004) Preparation of functionalized nanostructures on microchannel surface and their use for enzyme microreactors. *Chemical Engineering Journal,* **101**, 277-284.

Miyazaki, M., Kaneno, J., Yamaori, S., Honda, T., Briones, M.P.P., Uehara, M., Arima, K., Kanno, K., Yamashita, K., Yamaguchi, Y., Nakamura, H., Yonezawa, H., Fujii, M., Maeda, H. (2005) Efficient immobilization of enzymes on microchannel surface through His-tag and application for microreactor. *Protein and Peptide Letters*, **12**, 207-210

Miyazaki, M. and Maeda, H. (2006) Microchannel enzyme reactors and their applications for processing. *Trends in Biotechnology*, **24**, 463-470

Mao, H., Yang, T., and Cremer, P. S. (2002) Design and characterization of immobilized enzymes in microfluidic systems. *Analytical Chem*istry, **74**, 379-385

Nakamura, H., Li, X., Wang, H., Uehara, M., Miyazaki, M., Shimizu, H., Maeda, H. (2004) A simple method of self assembled nano-particles deposition on the micro-capillary inner walls and the reactor application for photo-catalytic and enzyme reactions. *Chemical Engineering Journal,* **101**, 261-268.

Nomura, A., Shin, S., Mehdi, O. O., Kaufmann, J. M. (2004) Preparation, characterization, and application of an enzyme-immobilized magnetic microreactor for flow-injection analysis. *Analytical Chemistry*, 76, 5498-5502.

Richter, T., Shultz-Lockyear, L. L., Oleschuk, R. D., Bilitewski, U., and Harrison, D. J. (2002) Bi-enzymatic and capillary electrophoretic analysis of non-fluorescent compounds in microfluidic devices determination of Xanthine. *Sensor and Actuators B,* **81**, 369-376

Sakai-Kato, K., Kato, M., Ishihara, K., and Toyo'oka, T. (2004) An enzyme-immobilization method for integration for biofunctions on a microchip using a water-soluble amphiphilic phospholipid polymer having a reaction group. *Lab on a Chip*, **4**, 4-6

Sakai-Kato, K., Kato, M., and Toyo'oka, T. (2003) Creation of an on-chip enzyme

reactor by encapsulating trypsin in sol-gel on a plastic microchip. *Analytical Chemistry*, **75**, 388-393

Schmid, A., Dordick J. S., Hauer, B., Kierner, A., Wubbolts, M., and Witholt, B. (2001) Industrial biocatalysis today and tomorrow. *Nature* **409**, 258-268

Schoemaker, H. E., Mink, D., and Wubbolts, M. G. (2003) Dispelling the myths-biocatalysis in industrial synthesis. *Science*, **299**, 1694-1697

Seong, G. H. and Crooks, R. M. (2002) Efficient mixing and reactions within microfluidic channels using microbead-supported catalysts. *Journal of the American Chemical Society,* **124**, 13360-13361

Sotowa, K.-I., Miyoshi, R., Lee, C.-G., Kang, Y., and Kusakabe, K. (2005) Mixing and enzyme reactions in a microchannel packed with glass beads. *Korean J. Chem. Eng*., **22**, 552-555.

Sotowa, K.-I., Takagi, K., and Sugiyama, S. (2008) Fluid flow behavior and the rate of an enzyme reaction in deep microchannel reactor under high-throughput condition. *Chem. Eng. J*., **135S**, S30-S36.

Strook, A. D., Dertinger, S. K. W., Ajdari, A., Mezic, I., Stone, H. A., and Whitesides, G. M. (2002) Chaotic mixer for microchannels. *Science*, **295**, 647-651.

Srinivasan, A., Wu, X., Lee, M.-Y., and Dordick, J. S. (2004) Bacterial P450-catalyzed polyketide hydroxylation on a microfluidic platform. *Biotechnology and Bioengineering*, **88**, 528-535

Szekely, L. and Guttman, A. (2005) New advances in microchip fabrication for electro-chromatography. *Electrophoresis*, **26**, 4590-4604

Tanaka, Y., Slyadnev, M. N., Sato, K., Tokeshi, M., Kim, H.-B., and Kitamori, T. (2001) Acceleration of enzymatic reaction in a microchip. *Analytical Science* **17**, 809-810

Tanaka, Y., Slyadnev, M. N., Hibara, A., Tokeshi, M., and Kitamori, T. (2000) Non-contact photochemical control of enzyme reactions on a microchip by using a compact diode laser. *Journal of Chromatography A* **894**, 45-51.

Thomsen, M. S., Plot, P., and Nidetzky, B. (2007) Development of a microfluidic immobilised enzyme reactor. *Chemical Communications*, 2527-2529.

Tokeshi, M., Minagawa, T., Uchiyama, K., Hibara, A., Sato, K., Hisamoto, H., and Kitamori, T. (2002) Continuous-flow chemical processing on a microchip by combining microunit operations and a multiphase flow network. *Analytical Chemistry*, **74**, 1565-1571

Qu H. Y., Wang, H. T,. Huang, Y., Zhong, W., Lu, H. J., Kong, J. L., Yang, P. Y., and Liu, B. H. (2004) Stable microstructured network for protein patterning on a plastic microfluidic channel: strategy and characterization of on-chip enzyme microreactor. *Analytical Chemistry*, **76**, 6426-6433

Urban, P. L., Goodall, D. M., and Bruce, N. C. (2006) Enzymatic microreactors in chemical analysis and kinetic studies. *Biotechnology Advances*, **24**, 42-57

Wang, H., Li, X., Nakamura, H., Miyazaki, M., and Maeda, H. (2002) Continuous particle self-arrangement in a long micro-capillary. *Advanced Materials* **14**, 1662-1666

Wang, H., and Holladay, J. D. (2005) *Microreactor Technology and Process Intensification*, *ACS Symposium Series, 914*, Washington, D. C.: American Chemical Society.

Watts, P. and Haswell, S. J. (2005) The application of micro reactors for organic

synthesis. *Chemical Society Review*, **34**, 235-246.

Wu, H. L., Tian, Y. P., Liu, B. H., Lu, H. J., Wang, X. Y., Zhai, J. J., Jin, H., Yang, P. Y., Xu, Y. M., and Wang, H. H. (2004) Titania and alumina sol-gel-derived microfluidics enzymatic-reactors for peptide mapping: design, characterization, and performance. *Journal of Proteome Research*, **3**, 1201-1209

Xue, Q., Wainright, A., Gangakhedar, S., and Gibbons, I. (2001) Multiplexed enzyme assays in capillary electrophoretic single-use microfluidic devices. *Electrophoresis* **22**, 4000-4007

Yamaguchi, Y., Ogino, K., Yamashita, K., and Maeda, H. (2003) Rapid micromixing based on multilayer laminar flows. *Journal of Chemical Engineering of Japan*. **37**, 1265-1270

Yamaguchi, Y., Ogino, K., Takagi, F., Honda, T., Yamashita, K., Miyazaki, M., Nakamura, H., and Maeda, H. (2005) Partial chemical modification of a microchannel and stabilization of water-oil phase separation. in *Proceedings of the 8th International Conference on Microreaction Technology (IMRET 8)*, 132w, New York: American Institute of Chemical Engineers.

Yoon, S.-K., Choban, E. R., Kane, C., Tzedakis, T., Kenis, P. J. A. (2005) Laminar flow-based electrochemical microreactor for efficient regeneration of nicotinamide cofactors for biocatalysis. *Journal of the American Chemical Society* **127**, 10466-10467

Zhao, B., Moore, J. S., and Beebe, D. J. (2002) Principles of surface-directed liquid flow in microfluidic channels. *Analytical Chemistry*, **74**, 4259-4268

Ziaie, B., Baldi, A., Lei, M., Gu, Y. D., and Siegel, R. A. (2004) Hard and soft micromachining for bioMEMS: review of techniques and examples of applications in microfluidics and drug delivery. *Advanced Drug Delivery Review*, **56**, 145-172