# A simple method for surface modification of microchannels

**Jun Kaneno,[a] Ryo Kohama,[a] Masaya Miyazaki,[b] Masato Uehara,[c] Kenichi Kanno,[a] Masayuki Fujii,[a] Hazime Shimizu[b] and Hideaki Maeda*[b]**

[a] *Department of Biological and Environmental Chemistry, Kyushu School of Engineering, Kinki University, 11-6 Kayanomori, Iizuka, Fukuoka 820-8555, Japan*
[b] *Micro-space Chemistry Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), 807-1 Shuku, Tosu, Saga 841-0052, Japan. E-mail: maeda-h@aist.go.jp; Fax: +81(942)81-3657; Tel: +81(942)81-3676*
[c] *Department of Applied Chemistry, Faculty of Engineering, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan*

***Received* (*in Montpellier, France*) *9th June 2003, Accepted 12th August 2003***
***First published as an Advance Article on the web 1st October 2003***



We previously developed a simple surface modification procedure to form a nanostructure on a microcapillary surfaces. However, only one set of conditions was examined and further optimization appeared necessary. This paper presents a detailed examination of the surface modification procedure and the effects of the surface modification level on the immobilisation of lipase. We first performed the reaction using a microcapillary with a 320 μm i.d and a 20 cm length. The number of surface amino groups was increased by increasing the content of 3-aminopropyltriethoxysilane in the silylating reagent by 60%, but a much higher content did not further increase the number of amino groups on the surface. The number of immobilised amino groups did not influence the amount of immobilised lipase. The performance of the microcapillary reactors was evaluated using the 7-acetoxycoumarin hydrolysis reaction. The microcapillary reactors showed equal reaction efficiency to each other, implying that surface structure, rather than the number of amino groups, affect microreactor performance. In a comparison of efficiency with a batchwise system, microreactors showed higher efficiency. We also applied our surface modification method to a ceramic microreaction device, which has a square channel (400 μm × 400 μm × 20 cm). The resulting lipase-immobilized ceramic microreaction device retained the same reaction efficiency. These results demonstrate that this modification method is applicable for the further development of microreaction devices.

## Introduction

Miniaturised chemical analysis and synthesis systems have attracted much interest recently.[1–6] Microchannel systems have several attractive features for chemical reactions such as: (1) rapid heat exchange, which cannot be achieved with a usual batchwise system; (2) rapid mass transfer; (3) mainly laminar flow and (4) large surface and interface areas.[6] Taking advantage of these features, several reaction devices have been reported to demonstrate potential applications. These include highly exothermal reactions,[7] *in situ* generation of hazardous compounds,[8] efficient solvent extraction[9] and a rapid energy transfer system.[10] Still, there are many potential applications for miniaturised synthetic reactors able to utilise small amounts of catalysts in conjunction with very limited volumes.

Immobilisation of catalysts on the surface of insoluble materials has served as a method for catalyst reuse. In a microreactor system, the microchannel wall surface area greatly exceeds that of typical batchwise systems. Several catalytic microreactors have been developed and their advantages have been demonstrated.[11,12] In addition, surface-modification methods of microchannels have been developed to increase the surface area. Zeolite-immobilised microreactors and porous silica microchannel devices have been applied for catalytic reactions, demonstrating higher reaction efficiencies.[13–16] For an enzyme reaction, Drott *et al.* utilized porous silica microchannel devices to create efficient enzyme microreactors.[15] However, these devices are complicated to prepare and appear unsuitable for routine production.

We have developed several microchannel surface modification methods.[17–19] By the orderly arrangement of nanoparticles,[17] we demonstrated that the reduction of methylene blue was enhanced in a titanium-coated nanoparticle-modified microreactor over that of a simple titanium-coated microchannel.[18] We developed a highly efficient enzyme microreactor by simple modification of a microchannel surface using a 1:1 (v/v) mixture of methyltriethoxysilane and 3-aminopropyltriethoxysilane.[19] However, only one set of modification conditions was used, which seems sub-optimal. In addition, because we only examined serine protease cucumisin for immobilisation, we seek to test the applicability of this method to other enzymes. The present study describes the detailed examination of our surface modification method. This time, we used lipase as the model case because this enzyme has been used in enzymatic processes. We also applied this method to a planar ceramic microchannel device, to examine whether this method is applicable to further development of efficient microreaction devices.

## Material and methods

### General

GL Science Co., Ltd. (Tokyo, Japan) provided a fused silica microcapillary. Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) supplied 3-aminopropyltriethoxysilane and methyltriethoxysilane. Wako Chemical Ind. (Osaka, Japan) and Sigma–Aldrich

Co. (St. Louis, MO, USA) supplied other reagents for surface modification. Lipase from *Candita Lugosa* was purchased from Sigma (St. Louis, MO, USA). Water was freshly prepared with a Milli-Q (Millipore Corp., Medford, MA, USA). Organic solvents were distilled prior to use. All solutions were filtered through a PTFE filter (0.45 μm) prior to use and loaded into 1 ml Hamilton gastight syringes (Hamilton Co., Reno, NV, USA). Solutions were supplied to the microreactor using a KDS 230 syringe pump with a parallel syringe holder (KD Scientific Inc., New Hope, PA, USA). HPLC analysis was performed by an Alliance 2965 system (Waters Corp., Medford, MA, USA) with a Wakopak C18AR column (3.0 × 250 mm) using a linear gradient of 0.05% TFA–acetonitrile over 60 min at a flow rate of 0.5 ml $min^{-1}$ at 30 °C. Each compound was confirmed by LC-MS (ABI Mariner ESI-TOF system).

### Surface modification of the microcapillary

A fused silica microcapillary (320 μm i. d. × 20 cm length) was used for microreactor preparation. The microreactor system was assembled as shown in Fig. 1. The capillary was connected with a Teflon tube by heat-shrink tubing (there is no enzyme absorption on the Teflon and heat-shrink tubes). The Teflon tube was connected to the syringe with a PTFE adapter (Flon Chemical Inc., Osaka, Japan). The microcapillary was treated with Piranha solution [7:3 (v/v) mixture of conc. $H_2SO_4$:30% $H_2O_2$] for 12 h at room temperature at a flow rate of 1.0 μl $min^{-1}$, followed by washing with water (total volume 1 ml). Then, the capillary was treated with 3% solution of various mixtures of 3-aminopropyltriethoxysilane and methyltriethoxysilane in 97% ethanol in water for 1 h at a flow rate of 1.0 μl $min^{-1}$ at room temperature. After washing with ethanol (at a flow rate of 10 μl $min^{-1}$, total volume 1 ml), the capillary was heated at 115 °C for 1 h. The number of amino groups immobilised on the microchannel surface was estimated by the modified Gisin's procedure.[20] The surface structure was analysed by scanning electron microscopy. FE-SEM image was obtained using a Hitachi S-5200 (Hitachi, Co., Tokyo, Japan). A sample was prepared by microcapillary breakage followed by platinum sputtering on the surface.

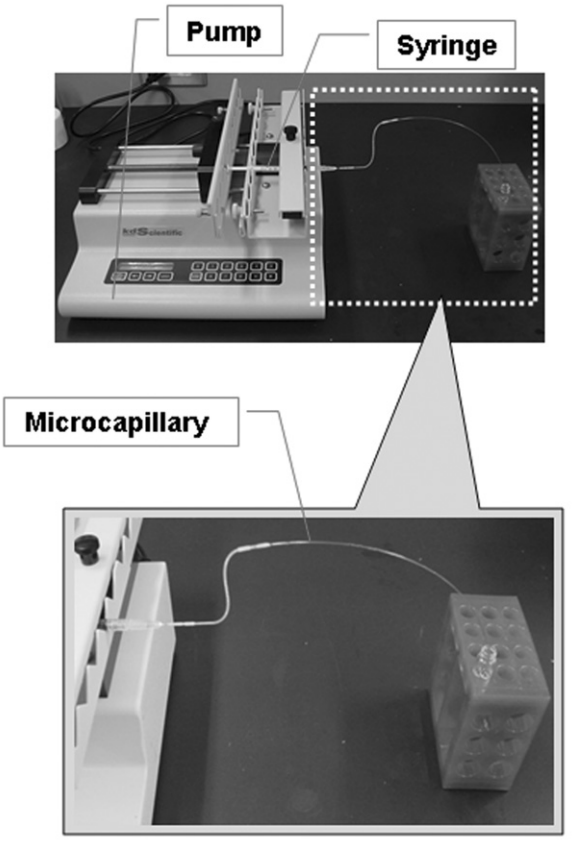


**Fig. 1** Capillary microreactor used in this study.

### Fabrication of the ceramic microreactor

The microchannel (400 μm × 400 μm × 20 cm) shown in Fig. 2 was mechanically fabricated on a ceramic plate (3 cm × 3 cm × 5 mm, obtained from Mitsui Mining Materials Co., Tokyo, Japan). Wet processing was performed in a Robodrill (Fanuc α14A, Yamanashi, Japan) equipped with a flat end mill (ϕ 400 μm). The microchannel size was confirmed using a Laser 3D profile microscope (Keyence Co., Osaka, Japan). The top glass plate assembly was achieved by baking (650 °C, 2 h). Inlet and outlet tubes were attached with inorganic adhesive.

### Surface modification of the ceramic microreactor

The microreactor system was assembled as follows. The inlet and outlet tubes of the ceramic microreactor were connected with a Teflon tube by heat-shrink tubing. The Teflon tube was connected to the syringe with a PTFE adapter. The microreactor was treated with Piranha solution for 12 h at room temperature at a flow rate of 1.0 μl $min^{-1}$, followed by washing with water (total volume 1 ml). Then, the microreactor was treated with 3% solution of various mixtures of 3-aminopropyltriethoxysilane and methyltriethoxysilane in 97% ethanol in water for 1 h at a flow rate of 1.0 μl $min^{-1}$ at room temperature. After washing with ethanol (at a flow rate of 10 μl $min^{-1}$, total volume 1 ml), the microreactor was heated at 115 °C for 1 h. The number of amino groups immobilised on the microchannel surface was estimated by the modified Gisin's procedure.[20]

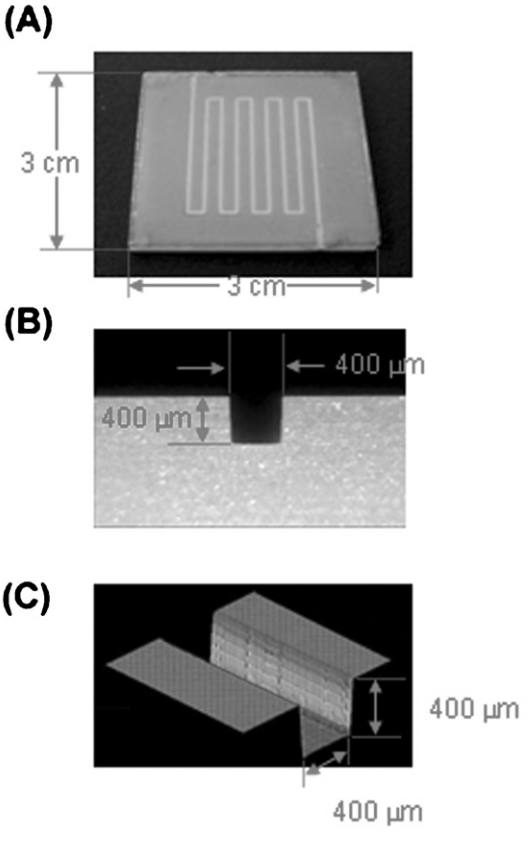


**Fig. 2** Fabricated ceramic microchannel reactor: (A) top view of the microreactor; (B) side view of the fabricated microchannel; (C) 3D profile of the microchannel obtained by laser microscopic analysis.

### Immobilisation of enzyme on the microchannel

The amino-functionalised microcapillary prepared above was treated with a 1 mM solution of succinic anhydride in DMF for 2 h at a flow rate of 5.0 μl $min^{-1}$ at room temperature to create the carboxyl function. After washing with DMF, the resulting carboxyl group was reacted with a 1 M solution of WSCI-HCl (1-ethyl-3(3-aminopropyl)carbodiimide hydrochloride) and *N*-hydroxysuccinimide in DMF for 1 h, followed by washing with DMF, water and PBS (30 min each). The surface-modified microchannel was filled with a PBS solution of lipase (5 mg $ml^{-1}$) and reacted for 12 h at 4 °C without pumping. After washing with PBS, the microcapillary was used for the hydrolysis reaction or estimation of the amount of immobilised lipase. The amount of immobilised enzyme was estimated by quantitative amino acid analysis. The lipase-immobilised microcapillary was subjected to acid hydrolysis using 6 M HCl. Amino acid analysis was performed by the standard PTC (phenyl isothiocyanate) procedure using these hydrolysed products. The amount of immobilised lipase was calculated by comparison with the amino acid composition of the lipase protein.

### Preparation of 7-acetoxycoumarin

Acetic anhydride (1.0 ml) was added to a stirred solution of 7-hydroxycoumarin (973 mg, 6.0 mmol), 4-dimethylaminopyridine (14.6 mg, 0.1 mmol), and triethylamine (126 μl, 9 mmol) in dichloromethane (20 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and then warmed to room temperature. The reaction was terminated by adding water after 3 h; the organic layer was washed with saturated NaCl solution and dried over $MgSO_4$. The crude product was obtained by filtration and evaporation; it was then purified by silica gel chromatography using dichloromethane. The yield was 919 mg (75%).

### Evaluation of the performance of the microreactor

The microchannel reactor prepared above was placed in an incubator and maintained at 35 °C. To this microreactor, a freshly prepared and filtered 1 ml solution of 7-acetoxycoumarin in PBS (100 μM, pH 7.4) was loaded using a syringe pump. The reaction was performed by continuous flow at constant pumping, at a flow rate of 32.2 μl (for the microcapillary) and 64.0 μl (for the ceramic microreactor) to fix the residence time at 0.5 min. The resulting solution was collected and analysed by HPLC. Each peak was confirmed by MS. The reaction yield was calculated from the peak area of the 7-hydroxycoumarin product. For the comparison of the performance with a batch-wise reaction, the residence time was fixed at 1 min. Therefore, the flow rates used were 16.1 μl (for the microcapillary) and 32.0 μl (for the ceramic microreactor) in this situation.

## Results

### Effect of changing the ratio of silylating reagent on the modification level of the microchannel surface

We have developed a simple method for the preparation of a functional nanostructure suitable for enzyme immobilisation on the microchannel surface. In the previous report, only a 50 : 50 (v/v) mixture of 3-aminopropyltriethoxysilane and methyltriethoxysilane was used.[19] This study examines the effect of the ratio of 3-aminopropyltriethoxysilane and methyltriethoxysilane on the level of surface modification. The modification was performed as shown in Fig. 3. First, we observed the surface structure using FE-SEM. As in our previous report,[19] the surface roughness increased in all cases (data not shown). Thus, the changes in ratio of silyation agents did not affect nanostructure formation.

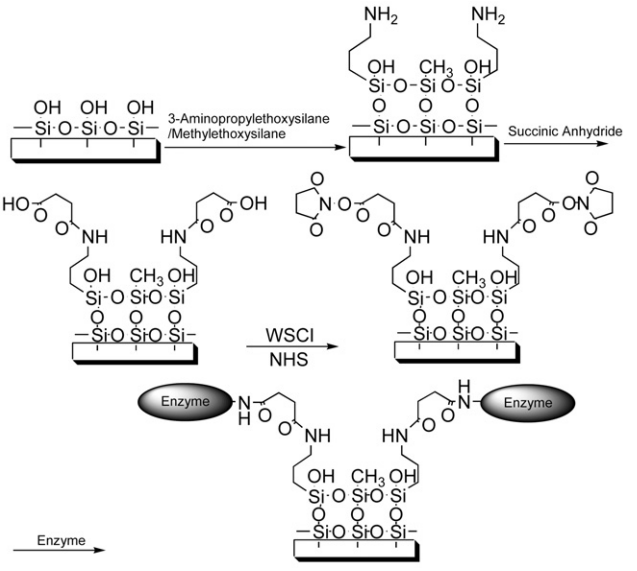


**Fig. 3** Strategy for surface modification of the microchannel.

The number of immobilised amino groups on the microchannel surface are shown in Table 1 for the microcapillary system. In all cases, several hundreds of amino groups were created on 1 $nm^2$ square of the surface. The number of amino groups increased in correlation with increasing amount of 3-aminopropyltriethoxysilane, from 20% to 60%, as expected. However, the number of surface amino groups did not increase further when 80% and 100% 3-aminopropyltriethoxysilane were used. We also examined the effect of total concentration of silylating agent by raising the total concentration from 3% to 5%, but the number of amino groups was unchanged (data not shown).

Next, we applied this surface modification procedure to the ceramic microreactor with a square channel. The modification was performed using the same procedure as for the microcapillary and the number of immobilized amino groups was titrated by Gisin's procedure. As shown in Table 2, the number of immobilized amino groups did not differ from that in the silica microcapillary.

### Effect of the modification level of the microchannel surface on microreactor performance

We prepared a lipase-immobilised microcapillary reactor using the amino-functionalised capillary, which was modified with 60% 3-aminopropyltriethoxysilane. Lipase molecule immobilisation was performed simply by loading reagents into the microchannel as shown in Fig. 3. With this capillary, we were able to immobilise 120 pmol of lipase. Based on the size of lipase, up to 12 pmol of enzyme molecule can be immobilized on the microchannel surface in a monolayer by close packing. Thus, lipase was immobilized as a multilayer and at least 10 layers exist in this microreactor.

First, we examined microcapillary reactor performance using these capillaries. The reaction was performed by supplying substrate solution by syringe pumping. Fig. 4 shows the

**Table 1** Surface modification level of the microcapillary (320 μm i.d.)

| % APS[a] | 20 | 40 | 60 | 80 | 100 |
|---|---|---|---|---|---|
| Amino groups $nm^{-2}$ | 381 | 515 | 991 | 905 | 1060 |
| Total amount of immobilised enzyme/pmol | 120 | 119 | 121 | 121 | 120 |
| Yield (%)[b] | 85 | 87 | 86 | 85 | 86 |

[a] Volume % of 3-aminopropyltriethoxysilane in total silylating reagent. [b] Yield at 0.5 min reaction in the microreactor. The residence time within the microcapillary was considered as the reaction time for the microreactor.

**Table 2** Surface modification level of the ceramic microreactor (400 μm × 400 μm)

| % APS[a] | 20 | 40 | 60 | 80 | 100 |
|---|---|---|---|---|---|
| Amino groups $nm^{-2}$ | 365 | 520 | 971 | 955 | 999 |
| Total amount of immobilised enzyme/pmol | 161 | 155 | 161 | 171 | 160 |
| Yield (%)[b] | 70 | 70 | 72 | 68 | 67 |

[a] Volume % of 3-aminopropyltriethoxysilane in total silylating reagent. [b] Yield at 0.5 min reaction in the microreactor. The residence time within the microchannel was considered as the reaction time for the microreactor.

improved reaction efficiency of the microreactor systems over that of the batchwise reaction using the same molar ratio of enzyme/substrate; the reaction was nearly completed within 1 min. This might result from the effect of a continuous-flow reaction in the microchannel, which continuously provides fresh substrate solutions, while the substrate concentration decreases as the reaction proceeds in the batchwise systems. Further studies are required to address the mechanisms.

We also examined the effects of the level of modification on enzyme immobilisation and microcapillary reactor performance. Table 1 shows that changes in the ratio of 3-aminopropyltriethoxysilane and methyltriethoxysilane did not alter microcapillary reactor performance or the amount of immobilised enzyme.

Next, we performed the same experiments in the ceramic microreactor with square microchannel. As shown in Table 2, we could immobilize approximately 160 pmol of lipase on the surface, which was unaffected by changes in the ratio of 3-aminopropyltriethoxysilane and methyltriethoxysilane. Also, the resulting lipase-immobilized microreactor showed similar performances as the microcapillary reactor (Fig. 4). Thus, the area of the surface nanostructure in the capillaries was not altered by changing the ratio of 3-aminopropyltriethoxysilane and methyltriethoxysilane. We could immobilise similar amounts of enzymes in each system. Also, an excess of amino groups in the nanostructured area did not affect enzyme immobilisation. Perhaps fewer amino groups were used for immobilisation of the enzyme and the remainder neither reacted nor interfered with the enzyme reaction.

## Discussion

Nanostructures have attracted interest as reaction apparatus, especially for catalytic reactions. A zeolite-immobilised microreactor and porous silica microreaction systems have been prepared.[13–16,21] However, these methods are difficult and seem to be unsuitable for commercial production of standardised microreactors. A previous study demonstrated that nanoparticles can be arranged on a microchannel wall by a relatively simple method.[17] However, technical difficulties remain, especially in the functionalisation of the surface for enzyme immobilisation. In the present study, we examined details of the surface modification level using a simple method to prepare a nanostructure on a microchannel surface that we developed.[19] The porous structure was observed by sol-gel entrapment of the enzyme on sintered glass.[22,23] In this report, we immobilised at least 10 layers of very large enzyme molecules on a microchannel surface. We also examined the effect of the surface modification conditions for lipase immobilisation. Because the enzyme is a huge molecule, the number of immobilised amino groups did not influence enzyme immobilisation. Rather, the surface structure is important. Immobilisation of small molecules such as organometallic compounds might be affected by changing the modification conditions.

In conclusion, we have developed a simple surface modification method for microchannels, which is applicable to both microcapillaries and square microchannels. We examined the microchannel surface modification level for a lipase-immobilised microreactor. The surface nanostructured microchannel reaction system might be useful for further development of efficient microreaction devices.

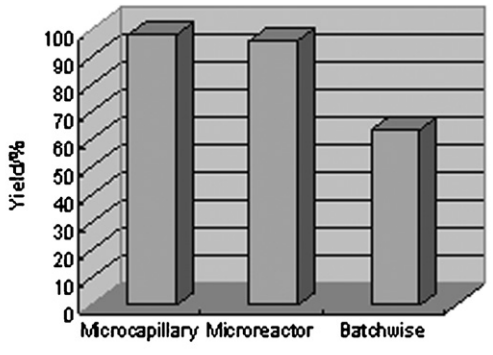


**Fig. 4** Comparison of the reaction efficiency of microreactors in a solution-phase batchwise reaction. The reaction was performed using 100 μM solution of 7-acetoxycoumarin for 1 min at 35 °C. For the microcapillary reactor (320 μm i.d. × 20 cm length) and ceramic microreactor (400 μm × 400 μm × 20 cm), the reaction was performed in the continuous-flow mode. Therefore, the residence time within the microchannel is considered as the reaction time for the microreactor. The flow rates used were 16 μl $min^{-1}$ (for the microcapillary) and 32 μl $min^{-1}$ (for the microreactor). In the batchwise reaction, the same ratios of reactor volume/enzyme amount as of microcapillary reactor was used.

## References

1 J. P. Kutter, *Trends Anal. Chem.*, 2000, **19**, 352.
2 D. J. Beebe, G. A. Mensing and G. M. Walker, *Annu. Rev. Biomed. Eng.*, 2002, **4**, 261.
3 S. H. DeWitt, *Curr. Opin. Chem. Biol.*, 1999, **3**, 350.
4 J. Yoshida, *Micromachines*, 2001, **34**, 13.
5 P. D. I. Fletcher, S. J. Haswell, E. Pombo-Villar, B. H. Warrington, P. Watts, S. Y. F. Wong and X. Zhang, *Tetrahedron*, 2002, **58**, 4735.
6 W. Ehrfeld, V. Hessel and H. Lowe, *Microreactors: New Technology for Modern Chemistry*, Wiley-VCH, Weinheim, 2000.
7 V. Hessel, W. Ehrfeld, K. Golbig, C. Hofmann, S. Jungwirth, H. Lowe, T. Richter, M. Storz, A. Wolf, O. Worz and J. Breysse, in *Proc. IMRET3*, ed. W. Ehrfeld, Springer, Berlin, 2000, p. 151.
8 T. M. Floyd, M. W. Losey, S. L. Firebaugh, K. F. Jensen and M. A. Schmidt, in *Proc. IMRET3*, ed. W. Ehrfeld, Springer, Berlin, 2000, p. 171.
9 M. Tokeshi, T. Minagawa, K. Uchiyama, A. Hibara, K. Sato, H. Hisamoto and T. Kitamori, *Anal. Chem.*, 2002, **74**, 1565.
10 J. M. Campbell, M. E. Domagala and C. T. Campbell, *J. Vac. Sci. Technol., Part A*, 1991, **3**, 1693.
11 K. Kusakabe, D. Miyagawa, Y. Gu, H. Maeda and S. Morooka, *J. Chem. Eng. Jpn.*, 2001, **34**, 441.
12 N. G. Wilson and T. McCreedy, *Chem. Commun.*, 2000, 733.
13 Y. S. S. Wang, J. L. H. Chau, A. Gavriilidis and K. L. Yeung, *Chem. Commun.*, 2002, 878.
14 E. V. Rebrov, G. B. F. Seijger, H. P. A. Calis, M. H. J. M. de Croon, C. M. van den Bleek and J. C. Schouten, *Appl. Catal. A.*, 2001, **206**, 125.
15 J. Drott, L. Rosengren, K. Lindstrom and T. Laurell, *Microchim. Acta*, 1999, **131**, 115.
16 F. Jones, Z. H. Lu and B. B. Elmore, *Appl. Biochem. Biotech.*, 2002, **98**, 627.
17 H. Wang, X. Li, H. Nakamura, M. Miyazaki and H. Maeda, *Adv. Mater.*, 2002, **14**, 1662.
18 X. Li, H. Wang, K. Inoue, M. Uehara, E. Abe, H. Nakamura, M. Miyazaki and H. Maeda, *Chem. Commun.*, 2003, 964.
19 M. Miyazaki, J. Kaneno, M. Uehara, M. Fujii, H. Shimizu and H. Maeda, *Chem. Commun.*, 2003, 648.
20 B. F. Gisin, *Anal. Chim. Acta.*, 1978, **58**, 248.
21 Y. Ma, W. Tong, H. Zhou and S. L. Suib, *Microporous Mesoporous Mater.*, 2000, **37**, 243.
22 M. T. Reetz, A. Zonta and J. Simpelkamp, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 301.
23 M. T. Reetz, A. Zonta, J. Simpelkamp and W. Konen, *Chem. Commun.*, 1996, 1397.