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Simple method for preparation of nanostructure on microchannel surface and its usage for enzyme-immobilization

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Received (in Cambridge, UK) 7th January 2003, Accepted 29th January 2003
First published as an Advance Article on the web 11th February 2003

We developed a novel preparation method of nanostructure on the microchannel surface formed by sol–gel like simple treatment with 3-aminopropyltriethoxysilane, which is suitable for a highly efficient enzyme-immobilized microchannel reactor.

Microreactors have become a new and promising technology in chemistry, chemical engineering, and biotechnology fields.[1–5] Advancement of microreaction technology during these years has demonstrated several advantages of miniaturized chemical systems including cost, throughput, and kinetics. Such features promote emphasis of research on miniaturized system fabrications and their applications toward development of functional materials and drugs.

Nanostructures have attracted interest as a reaction apparatus especially for catalytic reactions. Several methods have been developed to form nanoporous structure, such as zeolite.[6] Zeolite immobilized microreactor and porous silicon microreaction systems have also been prepared in microreactor systems.[7–10] However, these methods are difficult and seem to be unsuitable for commercial production of standardized microreactors.

The present study was designed to develop a simple method to form a nanostructure suitable for catalyst immobilization on a microchannel surface. We applied the sol–gel procedure, which has been utilized for porous structure preparation in batchwise systems.

A typical procedure for surface modification follows. Commercially available silica capillary (320 μm inner diameter and 20 cm length) was used for microreactor preparation. The microcapillary inner surface was washed with *Piranha* solution (H_2SO_4: 30% H_2O_2 = 7:3) for 12 h. After washing with pure water, the microchannel was filled with a 3% solution of a 1:1 mixture of 3-aminopropyltriethoxysilane and methyltriethoxysilane in 95 vol% ethanol in water; it was then set in a thermostated incubator controlled at 25 °C. After 1 h, the capillary was rinsed with ethanol, then dried and baked in a drying oven at 115 °C for 1 h. The number of immobilized amino groups was estimated by Gisin's procedure using picric acid; it was determined as 1×10^{17} amino group cm^{-2}, meaning that approximately 10 amino groups exist in a 1 $Å^2$ area.† This result suggests that a multilayer structure was formed by silane treatment. Thus, we analyzed the surface image of the microcapillary inner wall. Fig. 1 shows an unusual surface structure, seen on the inner wall; non-treated capillaries had no such structure (data not shown).‡

We obtained a nanostructured microcapillary by this method and examined its usage as an enzyme microreactor. The microchannel was treated with a 1M DMF solution of succinic anhydride to introduce carboxyl groups on the inner wall. After formation of an active ester by a 1M DMF solution of 1-ethyl-3(3-aminopropyl)carbodiimide hydrochloride and *N*-hydroxysuccinimicde treatment, a serine protease cucumicin[11] was immobilized by just passing a 1 mg ml^{-1} solution in phosphate buffered saline (pH 7.4) through this microchannel at a flow rate of 0.2 μl min^{-1} at 25 °C. The amount of immobilized enzyme, estimated from quantitative amino acid analysis of hydrolyzed products of this microcapillary, was 130 pmol. Calculating from the size of cucumicin, up to 13 pmol of enzyme molecule can be immobilized by closed packing on the microchannel surface as a monolayer. Thus, cucumicin was immobilized as a multilayer; at least 10 layers being present in this microreactor.§ This result also supports existence of nanostructures on the microchannel wall.

We examined reaction efficiency using this enzyme-immobilized microreactor. Hydrolysis of 100 μM phosphate buffered saline solution (pH 7.4) of Suc-Ala-Ala-Pro-Phe-*p*NA was used for evaluation. The reaction was performed in a thermostated incubator controlled at 25 °C. In the microreactor, reaction was completed at 250 μl min^{-1} pumping, meaning that

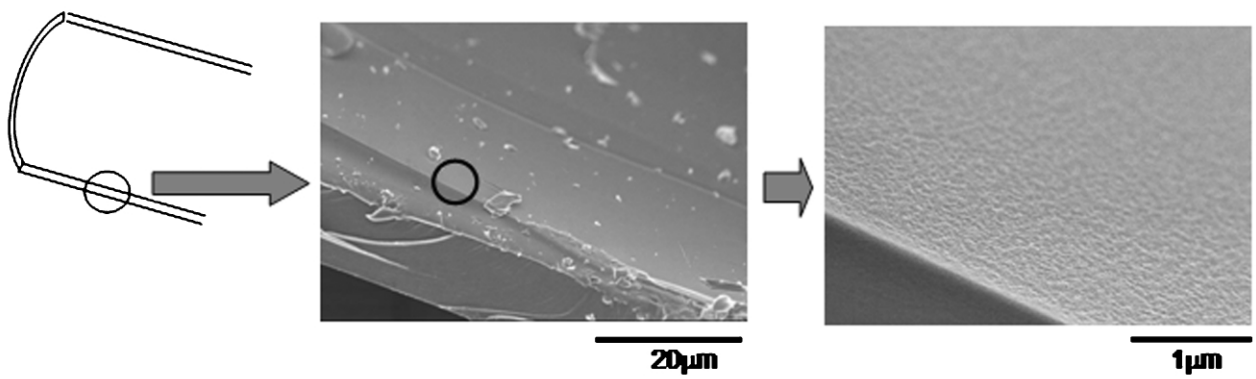

Fig. 1 FE-SEM visualization of a modified microchannel wall edge.

DOI: 10.1039/b212848a

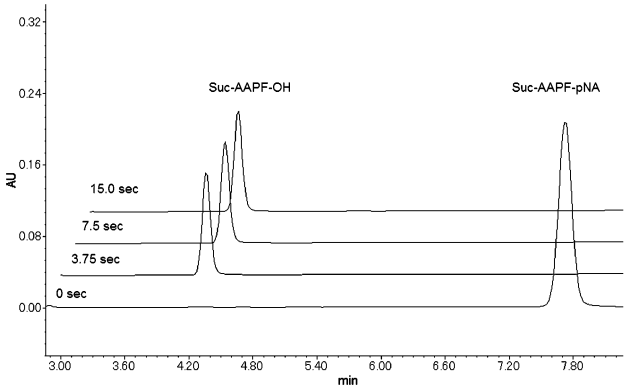

Fig. 2 Hydrolysis of Suc-Ala-Ala-Pro-Phe-*p*NA in a microchannel reactor. A 100 μM solution of substrate in PBS was charged into microreactor at 25 °C; product was analyzed by RP-HPLC. Residence time was considered to be the reaction time.

3.75 s or less was required to complete the reaction (Fig. 2). According to this flow rate, *ca.* 4 min. was required for 1 ml scale production. In a batchwise system using immobilized enzyme, several hours are required to complete a 1 ml scale reaction, although the same enzyme/substrate molar ratio as in the inside of microreactor was used and the mixture was vortexed at 1500 rpm (Table 1). Solution-phase batchwise reaction at the same enzyme/substrate molar ratio took more than 10 min to complete at a 1 ml scale. These results clearly demonstrated that the microreactor is an efficient reactor for enzyme reaction. Although the microreactor's small size would seem to preclude industrial scale production, with continuous operation, our microreactor can hydrolyze approximately 360 ml of 100 μM Suc-Ala-Ala-Pro-Phe-*p*NA per day; a single reactor can process 22.5 mg of Suc-Ala-Ala-Pro-Phe-*p*NA per day. This amount enables processing of approximately three tons per year with 1000 reactors. Therefore, the microreactor is capable of small quantity industrial scale production.

Table 1 Required time for hydrolysis of 1 ml of 100 μM solution of Suc-Ala-Ala-Pro-Phe-*p*NA by cucumicin at the same enzyme/substrate molar ratio

	Solution	Bead[a]	Microreactor
Time	15 min.	5 h	1 min.

[a] Cucumicin was immobilized on PEG-PS resin.

Nanostructures have garnered interest as a reaction apparatus, especially for catalytic reaction. In microreactor systems, zeolite immobilized microreactors[7,8] and porous silicon microreactors[9,10] have been prepared and used for catalytic reactions. However, these porous structures can only be prepared by a highly difficult method. In a previous report, we demonstrated that nanoparticles can be arranged on a microchannel wall by a relatively simple method.[12] An ordered porous structure can be obtained by this method. However, technical difficulties remain, especially in functionalization of the surface for enzyme immobilization. For the present study, we prepared a nanostructure tethering amino functional groups on a microchannel wall by a simple method. Porous structure was observed by sol–gel entrapment of enzyme on sintered glass.[13,14] In this report, we cannot conclude the structure is porous. However, we could immobilize huge enzyme molecules in at least 10 layers, indicating the nanostructure could be porous. To prove this, further studies are required. Also, although the detailed mechanism of enhanced reaction efficiency of microreactors remains unclear, our method might be useful for further development of a catalyst immobilized microreactor, especially for enzyme catalysts.

In summary, we have developed an enzyme-immobilized microchannel reactor with nanoporous surface structure formed by a solution of 3-aminopropylsilane. This nanostructure may also be of interest in the case of traditional metal catalysts and organometallic catalysts. Further studies on the mechanism of improved reaction efficiency of microreactors and application of nanostructure on the microchannel surface to other catalysts are in progress.

We thank Professor H. Yonezawa and Dr K. Arima for providing cucumicin enzyme.

Notes and references

† Quantitative amino acid analysis was performed by the standard PTC procedure. The amount of cucumicin was calculated by comparison with the amino acid composition of cucumicin protein.

‡ FE-SEM image was obtained using a Hitachi S-5200. A sample was prepared by breaking a microcapillary as shown in Fig. 1 followed by platinum spattering.

§ Immobilized cucumicin retained its full activity more than one month.

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