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Fabrication of a polystyrene microfluidic chip coupled to electrospray ionization mass spectrometry for protein analysis

Xianqiao Hu[a,b,c,*], Yuanyuan Dong[b], Qiaohong He[b], Hengwu Chen[b], Zhiwei Zhu[a,c]

[a] China National Rice Research Institute, Hangzhou 310006, China
[b] Institute of Microanalytical Systems, Department of Chemistry, Zhejiang University, Zijin'gang Campus, Hangzhou 310058, China
[c] Laboratory of Quality & Safety Risk Assessment for Rice (Hangzhou), Ministry of Agriculture, Hangzhou, China

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ABSTRACT

A highly integrated polystyrene (PS) microfluidic chip coupled to electrospray ionization mass spectrometry for on-chip protein digestion and online analysis was developed. The immobilized enzymatic microreactor for on-chip protein digestion was integrated onto microchip via the novel method of region-selective UV-modification combined with glutaraldehyde-based immobilization. The micro film electric contact for applying high voltage was prepared on chips by using UV-directed electroless plating technique. A micro-tip was machined at the end of main channel, serving as the interface between microchip and mass spectrometric detector. On-chip digestion and online detection of protein was carried out by coupling the microchip with mass spectrometry (MS). The influences of methanol flow rate in side channel on the stability of spray and intensity of signals were investigated systematically. Also the influence of sample flow rate on the performance of immobilized enzymatic reactor were investigated. Stable spray was obtained at the spray voltage of 2.8–3.0 kV and the methanol flow rate of 500–700 nL min^{-1} with the relative standard deviation (RSD) of total ion current (TIC) less than 10%. The influence of sample flow rate on the performance of immobilized enzymatic reactor was also studied. The sequence coverage of protein identification decreased with the increase of flow rate of the sample solution. A sequence coverage of 96% was obtained with immobilized enzymatic reactor at the sample flow rate of 100 nL min^{-1} with the reaction time of 8.4 min. It could detect cytochrome *c* as low as 10 μg mL^{-1} with the developed system. No obvious decrease in protein digestion efficiency was observed after the chip continuously performed for 4 h and stored for 15 d.

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1. Introduction

Proteomics is one of the most important research topics in the 21st century [1–3]. Mass spectrometry shows great potential in proteomics due to its sensitivity, quantitative and qualitative determination of biological macromolecules. However, protein digestion using proteases, which is the key sample preparation for protein analysis, is needed before it is introduced into mass spectrometry. Protein digestion is traditionally performed in solution. It suffers issues such as long digestion time, auto-digestion subproducts, poor enzyme-to-substrate ratio and low proteolytic digestion efficiency. Meanwhile, enzymes immobilized on a support possess advantages such as high digestion, low auto-digestion and reusablity [4,5]. More importantly, it opens the way to coupling protein digestion unit with mass spectrometric detection unit. Many works on immobilized enzymatic reactors were reported [5–8]. Lee et al. [9] developed a solid-phase bioreactor in microchannel by covalently attaching trypsin to UV-modified surface of PMMA channel using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS) as coupling reagents. The microreactor produced a sequence coverage for protein identification of 97% in a reaction time of approximately 20 s. Hence, it is feasible to combine the microchip immobilized with enzymatic reactor with mass spectrometry, reducing the workload. With such system, online protein digestion and online MS analysis could be implemented. However, no work on immobilizing enzyme on the polystyrene (PS) chip had been reported.

Microchips have been developed in the last decade and used for analyses of protein [10–13]. The interface connecting microchips with MS was a critical unit for the microchip–MS system. Electrospray ionization (ESI) can produce charged ions direct from liquids, simplifying online coupling with other units. The development

* Corresponding author at: China National Rice Research Institute, Hangzhou 310006, China. Tel.: +86 571 63370351; fax: +86 571 63370351.
E-mail address: hxhxqia@aliyun.com (X. Hu).

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of nano-ESI decreased sample consumption ranging from tens to hundreds nanoliters, and improved the compatibility between ESI–MS and microfluidic chips. Reviews on the approaches for coupling microfluidic chips to ESI–MS have been published [14–17]. There are mainly two approaches for the interfaces connecting microchips with ESI–MS. One is to insert external electrospray capillaries into the end channel of chip as interfaces [18,19]. It was widely used because of the excellent stability of spray, the mature and convenient technology to prepare electrospray capillaries. However, dead volumes arose from the interface were ineluctable. Moreover, it is labor intensive to attach external electrospray capillary, and the glue used in attaching interfaces might dissolve in organic solvents and produce interfering peaks afterward. The other one is to prepare integrated interfaces at the end of channel on microchips. It could overcome the problem of dead volumes arose from the former method effectively. Two types of integrated ESI–MS interfaces were developed, edge of chip [20,21] and chip-integrated ESI-tip [22,23]. Spraying from edge of microchip met the problem of relative large Taylor cone [14]. Generally, sprays with chip-integrated ESI-tips are stabler than those using edge of chip. However, procedures for chip-integrated ESI-tips are more complicated than those for the former ones. The polymer chips are more easily to be machined, compared with glass or silica chips. In this paper, micromachining method was applied to prepare PS chips with chip-integrated ESI-tips.

Electric contact, which is used for applying high voltage, is another important function in the microchip–ESI–MS system. It's an easy method to insert an electrode in reservoir to provide high voltage [20,24,25]. However, a high-voltage field generates in channel by using this method, interfering the analysis. Besides, the inserted electrodes reduce the integration level of whole system to a large extent. Integrated electrodes prepared mainly by using deposition/lithography/wet chemical etching technique [26], carbon inking [27] or low-melting-point alloy (LMA) [28] were developed. These methods are quite complicated, and the prepared electric contacts suffered the risk of delamination. Hu et al. [29] reported a novel method for the fabrication of gold microelectrodes on PS sheet by UV-directed electroless plating. The microelectrodes prepared by this simple method showed strong adhesion to substrates, good stability in strong acidic and alkaline solutions, and excellent accuracy in size. However, the UV-directed electroless plating method has never been applied for preparing electric contacts for microchip–ESI–MS system.

In this paper, a highly integrated PS microchip for on-chip protein digestion and online analysis was developed. First, we developed a new method for on-chip enzyme immobilization via region-selective UV-modification combined with glutaraldehyde-based immobilization. Then, the microchip immobilized with enzymatic reactor was connected to mass spectrometry with a micro-machined tip as interface. The electric contact for applying high voltage was prepared on the chip by using UV-directed electroless plating. The developed highly integrated microchip–ESI–MS system was demonstrated for on-chip digestion and online MS detection of cytochrome *c*.

2. Materials and methods

2.1. Chemicals and apparatus

The polystyrene sheets were purchased from Hangzhou Baiersi Plastic Co. (Hangzhou, China); an ion trap mass spectrometer (LCQ DECA XP, Thermo-Fisher Scientific Inc., MA, USA) and Voyager de pro MALDI–MS (Applied Biosystems, USA) were used for protein analysis; a 30 W low-pressure mercury lamp was employed for UV-pretreatment of PS sheets; Extec 10248 milling machine was a product from Extec Co. (USA); Model C Carver thermal system was bought from Carver Co. (USA).

2.2. Fabrication of the microfluidic chip integrated with immobilized enzymatic reactor, gold electric contact and ESI–MS emitter tip

The microfluidic chip coupled to ESI–MS was consisted of three functional modules: immobilized enzymatic reactor for on-chip protein enzymatic digestion, gold electric contact for applying high voltage, emitter tip for interface between chip and ESI–MS. The three functional modules were prepared systematically. The whole procedure for fabrication of integrated microfluidic chip was shown in Figs. 1 and 2.

2.2.1. Fabrication of integrated electric contact and microchannels

The micro channel network in a silicon mold was hot embossed into 4 cm × 2 cm PS substrate by using thermal system. The embossing process was operated at the temperature of 95 °C and the pressure of 3.27 MPa for about 5 min. After de-molding, two holes of 1/32 in. i.d. were drilled at two terminals of channels on PS sheet. The embossed channel network consisted of one main serpentine channel (20 cm) and one side channel (7 mm). All the channels were 60 μm in the bottom width, 110 μm in the top width and 50 μm in depth.

The gold film electric contact with desired configuration was prepared onto the PS cover sheet of 4 cm × 3 cm via UV-assisted region-selective electroless plating [29]. Briefly, PS cover sheet was firstly selectively modified with UV-lights through a photo-mask, and treated sequentially with ethylenediamine solution (containing 0.36 mol L^{-1} ethylenediamine and 50 mmol L^{-1} EDC) for 3 h, 1 mmol L^{-1} $HAuCl_4$ solution for 2.5 h and 0.1 mol L^{-1} $NaBH_4$ solution for 10 min to form gold nano-particle catalysts. The activated PS sheet was then placed into a gold plating bath (containing 0.125 mol L^{-1} Na_2SO_3, 0.6 mol L^{-1} HCHO, and 8 mmol L^{-1} $Na_3Au(SO_3)_2$) for about 30 min under room temperature. Finally, the PS sheet with gold film electric contact was subjected to a thermal treatment (80 °C for 3 h) to strengthen the adhesion of deposited gold film.

2.2.2. Fabrication of preliminary ESI–MS emitter tip

PS substrate sheet with channel network and PS cover sheet with gold film electric contact were cut along the dotted lines as shown in Fig. 1a and b. Then the cut substrate and cover sheets (Fig. 1c and d) were aligned and clamped, with channel network facing gold film. After that, the cut surfaces of clamped sheets were milled with sandpaper of 500 grit at the speed of 100–150 r min^{-1} till the two cut surface were in the same flat surface, followed by polished with sandpaper of 2000 grit at the same speed for about 10 min. The formed tip was the preliminary ESI–MS emitter. During milling and polishing, running water was supplied to cool down the chip.

2.2.3. Amination of the microchannels

PS substrate sheet with channel network and PS cover sheet with gold electric contact were treated with UV-lights for 2 h through designed photo-mask with channel network (Fig. 1e and f), causing carboxyl groups formed on designed surface of PS channel (Fig. 2a and b). Then the two PS sheets were treated with ethylenediamine solution for 3 h. Hence, the carboxylic groups formed in UV-irradiated region were aminated with ethylenediamine, leaving free amine groups of ethylenediamine which were grafted to the surfaces of PS channel (Fig. 2c).

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