A disposable poly(methylmethacrylate)-based microfluidic module for protein identification by nanoelectrospray ionization-tandem mass spectrometry

The design, fabrication, and analytical use of a poly(methylmethacrylate) (PMMA)-based microfluidic module for nanoelectrospray ionization-tandem mass spectrometry (nano-ESI-MS/MS) were described. The microfluidic module can be mass-produced at low costs and used as a disposable device to generate nano-ESI-MS/MS signals for protein identification from low amounts of protein samples. Compared with commercially available nanospray capillary tips, the module gave comparable signal quality and also offered advantages in convenience and easiness of operation, permitting repeated usage, and disposability.

Keywords: Poly(methylmethacrylate) / Disposable microfluidic module / Protein identification / Proteomics / Nano-electrospray ionization / Tandem mass spectrometry

1 Introduction

The advance of the human genome project has shown a great deal of promise in the biomedical research field. Within a few years, complete human genome sequence databases will be available to scientists for understanding disease mechanism, diagnosis, and new drug development [1]. While how the genomic sequence information will be exploited is still under intensive consideration, it is obvious now that, by using state-of-the-art mass spectrometry (MS) methods, proteins of interest to biomedical researchers can be mapped into sequence databases to find their identities [2]. This opens the possibility for systematic identification of the complete set of proteins that is expressed by the entire genome by a group of cells, that is, the proteome. Proteome is a linguistic equivalent to the concept of genome. Proteomic research, or proteomics, refers to the studies of the proteome itself and the functional analysis of the proteins using technologies of large-scale protein identification [3–5].

To achieve large-scale protein identification, it has been speculated that microfabricated devices may provide an effective alternative solution to other means [6]. Microfabrication offers at least two major noticeable advantages, miniaturization and integration, and is therefore considered to be able to mass-produce microdevices at low costs to perform large-scale chemical analysis [7]. Microfabricated microfluidic chips have been proposed to carry out chemical reaction, separation, and identification, and „lab-on-a-chip“ is termed to describe such a microfluidic system [6]. Recently, it has also been noticed that microfluidics for manipulating liquid solution is highly compatible with the contemporary efforts to fabricate a miniaturized electrospray (ESI), or a nanoelectrospray (nano-ESI), ion source to be used for MS [8]. A few dozen papers have been published [9–30] and reviewed [6] to describe various microfluidic devices in conjunction with ESI-MS or nano-ESI-MS systems.

Most of the microfluidic devices for ESI-MS reported to date used glass or quartz substrates [9–22]. The microfluidic channels of the glass/quartz microdevices were typically fabricated by photolithographic, wet chemical etching, and cover bonding procedures [11]. There have been a few reports describing the application of polymer materials, such as parylene [23], polycarbonate [24–26], poly(dimethylsiloxane) (PDMS) [27, 28], and poly(methylmethacrylate) (PMMA) [29, 30] to microfabricated ESI-MS devices. The microfabrication methods for the polymer substrates varied, ranging from simple knife cutting [30], replica molding [27, 28], UV laser machining [24–26], X-ray lithography [29], to a complicated micromachining process on a silicon/parylene composite substrate [23]. In this report, we describe a PMMA-based microfluidic module fabricated by a hot-embossing process using a quartz master template. The microfluidic module can be mass-produced at low cost and used as a disposable device to generate nano-ESI-MS signals for protein identification from small amounts of protein samples.
2 Materials and methods

2.1 Chemicals and materials

Methanol (HPLC grade) was purchased from Mallinckrodt (Phillipsburg, NJ, USA). Formic acid was purchased from Riedel-de Haën (Seelze, Germany). Bovine serum albumin (BSA), fibrinopeptide A (1536.6 Da), osteocalcin fragment 7–19 (1407.6 Da), bradykinin (1060.2 Da), LMYPTYLK (1028.3 Da), and VGGYGYGAK (871.0 Da) were purchased from Sigma Chemical (St. Louis, MO, USA). All reagents were of the highest grade available and were used directly without further purification. CE water was deionized distilled water that was filtered through a Barnstead E-pure system. The resistance of the water was more than 18.0 MΩ cm. Both the buffer and sample solutions were filtered through a 0.22 μm membrane before chip electrophoresis. NanoES capillaries (catalog ES381) were purchased from Protana (Odense, Denmark).

2.2 Fabrication of the nano-ESI microfluidic module

The configuration of the microfabricated device (Fig. 1) includes a cross microchannel and a fused-silica tubing with a silver glue-coated nano-ESI tip. The cross microchannel was fabricated using a hot embossing method [31, 32] on PMMA plexiglas substrate of approximately 2 cm in width × 4 cm in length and 1.5 mm thick. Briefly, the microchannels on master templates were formed by the combination of metal etch mask and wet chemical etching. A commercial blank photo mask substrate (Nanofilm), consisting of three layers (1 μm photoresist, 1 μm Cr, and 2.3 μm quartz, respectively) was used as a master template on which microcapillary channels were fabricated. The image on the film is first transferred to photoresist (PR) by standard lithography using a transparent film as a mask. After PR is developed, the blank photomask is immersed in Cr etchant to transfer patterns to on the Cr layer. Lastly, the residual PR is stripped, revealing the transferred image on the Cr layer, which will be used as the etch mask for further quartz etching. Microfluidic channels on the quartz substrate were formed by the combination of metal mask (Cr) and buffered oxide etchant (BOE, NH₄F:HF = 6:1) at room temperature to fabricate capillary channels with a smoother appearance. Quartz was etched in all the areas surrounding the channels, and the resulting structure was the inverse-raised three-dimensional image of the channels (size 80 μm × 40 μm). The microchannels were imprinted by pressing a quartz master template onto the top of a PMMA blank substrate. The entire device was heated at 130°C for 15 min. Once the microchannels were formed, a Ni wire (400 μm OD) was placed in the end of the separation channel to imprint a fitting channel for capillary tubing (Polymicro Technologies, Phoenix, AZ, USA; 365 μm OD and 50 μm ID) at the same imprinting temperature. The resulting PMMA plate with microfluidic channels and the capillary tubing was then clamped with another PMMA cover plate to form sealed channels with the attached capillary tubing. The PMMA devices were heated at 100°C for at least 8 min for bonding. Prior to bonding, four through holes (1 mm in diameter) were drilled on the cover plate as analyte and sample reservoirs. The bonding strength of the chips was estimated to be about 1.0 MPa using home-made tensile test equipment. The UV glue was applied to fill the dead volume between the microchip and the fused silica tubing. The tapered nano-ESI tip was formed by pulling the capillary against gravity with a weight of 33 g while a small section of the capillary was heated at 1300°C by a n-butane torch. The resulting tip was about 80 μm OD and 18 μm OD. Once the tip was formed, silver conductive glue (Electrolube Limited, Wargrave Berkshire, England) was painted on the surface of the tip by immersing the tip into glue and applying a gas stream or suction vacuum through the capillary.

2.3 Sample preparation, injection, and electroosmotic pumping

Protein and peptide standards were dissolved in 50% methanol and 0.1% formic acid before analysis. One power supplier (CZE 1000R; Spellman, Hauppauge, NY, USA) was utilized to provide the loading and electroosmotic voltages, and the power switching was done manually when the injection was needed. For continuous infusion mode, 6 kV was applied to the sample reservoir I. For injection mode, the sample loading was performed by...
applying 6 kV to sample reservoir I for 0.2 min, then switching the 6 kV manually to reservoir II for electroosmotic pumping. The buffer solution was composed of 50% methanol and 1% formic acid.

2.4 nano-ESI-MS/MS

All the data were acquired using an API 365 triple quadrupole mass spectrometer (PE Sciex, Thornhill, Canada) equipped with a home-built source housing to host nano-ESI microfluidic modules. For comparison purposes, some data were obtained using a commercially available nanospray capillary tip from Protana. A mini camera (model AVC 556N/F36(B); Hung-Li-Sun Co., Taipei, Taiwan) was installed inside the source housing to monitor spraying and the position of microfluidic module. An x-y-z translational stage was used to move the module for positioning. Low-energy collision-induced dissociation (CID) experiments were performed using nitrogen (CID gas valve was set to 3) as the collision gas and an optimized collision energy of 35 eV. Other instrument parameters (such as orifice, ring, and quadrupole voltages) were optimized the automatic tuning function provided by the API 365. The step size was set to 0.25 u and dwell time was set to 1 ms.

3 Results and discussion

3.1 The polymer-based nano-ESI-MS microfluidic module

Figure 1 shows a diagram for the polymer-based nano-ESI-MS microfluidic module and its utility in protein identification. The microfluidic channels were formed with PMMA substrate using hot embossing by quartz master and the channels were closed by thermally bonding a covering PMMA plate. A pulled fused-silica capillary coated with silver glue on the tip was inserted to the exit end of the channel and fixed with UV glue. Peptide samples can be loaded onto the module through sample reservoir I, and electroosmotically pumped to the nano-ESI emitter to generate peptide ion signals.

In our laboratory, we regularly use commercially available nanospray tips (from New Objective, Cambridge, MA, USA, or Protana, Odense, Denmark) to generate nano-ESI ions from trypsinized protein samples excised from an electrophoresis gel. These tips are usually made of glass, quartz, or fused silica. In a typical situation, a protein spot of interest was recognized from visualized protein patterns by contrasting the differences between case and control electrophoresis gels. The spot was excised, digested by trypsin, and desalted by a reversed-phase solid-phase extraction trap (ZipTip; Millipore, Boston, MA, USA). The resulting solution containing tryptic peptide fragments was sprayed into a tandem mass spectrometer using the commercial nanospray tips to yield product ion scan mass spectra. The MS/MS data were used to search the sequence database to find out the identity of the protein of interest. The polymer-based nano-ESI-MS microfluidic module can be used in a similar manner. The next section is a comparison of signal quality between those generated by a commercial available nanospray tip and a microfluidic module fabricated in our laboratory.

3.2 Signal quality

Figures 2A and B are full-scan mass spectra obtained for a 10 pmol/µL bradykinin standard solution using a microfluidic module fabricated in house and a Protana nanospray tip.

![Figure 2](image-url)
The total ion current and ion current at m/z 531 ([M+2H]++) were plotted against time for both (A), (C) the Protana nanospray tip and (B), (D) the microfluidic module, when they were tested using a 10 pmol/µL bradykinin standard solution.

The ion current at m/z 531 ([M+2H]++) resulted from five consecutive injections of 3 µL of 10 pmol/µL bradykinin standard solution.

Both mass spectra have comparable noise levels with major peaks at the same m/z value, 531.1, corresponding to doubly-protonated bradykinin, [M+2H]++. The singly-charged ion ([M+H]+, m/z 1061.2) is barely visible in Fig. 2A or B. The signal intensities of the two base peaks are about the same. Consequently, both devices give comparable signal-to-noise ratios for the bradykinin standard solution. The signal stability was also compared. In Fig. 3, the total ion current and ion current at m/z 531 are plotted against time for both the Protana nanospray tip and the microfluidic module. Both devices were able to generate stable ion signals for more than 20 min.

Figure 4 shows that five consecutive injections were performed manually and their signals were recorded. The signal intensity varied less than 20% between injections. The variation could be reduced if automatic injections were used. The module was able to generate signals from consecutive injections, indicating that it could be integrated with other microfluidic modules to perform multiple analyses sequentially. In Fig. 5, a mixture containing four peptides was analyzed and the results are shown for the analyses done with (A) a Protana nanospray tip and (B) the microfluidic module.

The signals that appear in Fig. 5A are better than some peaks in Fig. 5B. The signals at m/z 436, 531, and 768 in Fig. 5A and B are comparable, while the signals at 515, 871, and 1028 in Fig. 5B are significantly smaller.
3.3 Comparison with commercially available nanospray tips

In comparison with the commercial nanospray tips, the polymer-based microfluidic modules were used differently in several ways, even though they shared some common features such as providing an interface between electrophoresis and MS and both consumed about 1–3 μL of sample solution for each analysis. Firstly, pressurizing the sample solution with compressed air was usually required to initiate a stable nano-ESI signal the commercial tips. Frequently, it was also necessary to “break” the fine tip to start the nano-ESI, which caused difficulties in generating reproducible results and required experienced users. The sample solution was, by necessity, loaded into the capillary tip by centrifugation. In contrast, when the microfluidic module was used, analyte solution was transferred to sample reservoir I by a micropipette and electroosmotically pumped to the nano-ESI emitter, eliminating the tedious procedures of centrifugation, air-pressurization, or breaking the fine tip. Secondly, the microfluidic module was found to be useful for multiple repeated usages, more than 100 times without any noticeable cross-contamination. In contrast, the commercial nanospray tips can only be used for one sample since the tip end was “broken” and failed to load another sample by centrifugation.

3.4 Protein identification by searching sequence database

Figure 6 shows an MS/MS spectrum and the corresponding sequence tag generated from an SDS-PAGE-separated protein, BSA. The protein band was silver-stained, excised, and subject to trypsin digestion. The resulting mixture was desalted by ZipTip and analyzed by the microfluidic module. Although the signals were not strong, they could be used to deduce a sequence tag and the sequence tag was used to search the PIR protein sequence database using software provided by PE SCIEX. The corresponding sequence tag was (592.2) (I/L)EN(951.4) which was mapped to the correct protein sequence in the database.

4 Concluding remarks

A PMMA-based microfluidic module fabricated by a hot-embossing process using a quartz master template and by attaching a silver-coated pulled fused-silica nano-ESI emitter was described in this report. The use of a quartz master template and hot-embossing, and also the emitter being attached by UV-glue, made it possible to mass-produce these microfluidic modules at low cost. The analytical utility of the microfluidic module was compared with a commercially available nanospray tip. It was concluded that they yielded comparable signal quality but the microfluidic module provided certain advantages such as convenience and ease of operation, and permitting repeated usages without serious cross-contamination. Because of its potential in cutting manufacturing costs in mass production, it can be used as a disposable device after certain numbers of analyses, or for trace analysis where analyte carry-over could potentially become a problem. Both the inputs and the outputs of microfluidic modules are liquids, so integration is readily achieved by modular designs and fabrication. With the goal of making microfluidic devices to useful tool in proteomic research, addi-
tional investigations are being carried out in our labora-
tory to expand the function of the microfluidic module,
including (i) an on-chip trypsin reactor module, (ii) a de-
salting module, and (iii) a frontal immunoaffinity-based
extraction module.

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