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Journal of Chromatography A, 924 (2001) 315–322

JOURNAL OF
CHROMATOGRAPHY A

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On-line coupling of micro-enzyme reactor with micro-membrane chromatography for protein digestion, peptide separation, and protein identification using electrospray ionization mass spectrometry

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Abstract

To miniaturize high-performance membrane chromatography, a poly(vinylidene fluoride) membrane medium, employed as the stationary phase, is sandwiched between two poly(dimethylsiloxane) substrates containing the microchannels. The microchannels are fabricated by the capillary molding technique, involving the use of capillaries as the channel template and the fluid inlet/outlet. The micro(μ)-membrane chromatography system is coupled with a μ-enzyme reactor containing immobilized trypsins for performing rapid protein digestion, peptide separation, and protein identification using electrospray ionization mass spectrometry. Separation performance of cytochrome c digest in μ-membrane chromatography is compared with the results obtained from a regular reversed-phase μ-liquid chromatography. The efficacy and the potentials of μ-membrane chromatography in tryptic mapping are reported. On-line integration of the μ-enzyme reactor with μ-chromatographic separation techniques and electrospray ionization mass spectrometry clearly provides a microanalytical platform for automated sample handling, minimized sample loss, and reduced sample consumption. It also provides enhanced detection sensitivity and dynamic range for the analysis of complex protein mixtures such as cell lysates in proteomics research. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Membrane chromatography; Stationary phases, LC; Proteins; Peptides; Cytochromes

1. Introduction

There is great interest in miniaturized analytical systems for life science research, clinical laboratories, high throughput drug screening, quality control of biopharmaceuticals manufacturing, and environmental monitoring. Through micro(μ)- and nano-fabrication, either single or multiple, integrated, self-contained laboratories of a size equivalent to a computer chip can be constructed in the near future. It is possible that these systems will be capable of executing analyses in which biochemical reactions, separations, and various forms of detection are integrated into a single or complex microfluidic network.

The area of microfluidics that includes a separation component has expanded rapidly during the past decade [1–9]. In most cases, the separations are accomplished electrophoretically with an applied electric potential. To incorporate chromatographic

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PII: S0021-9673(01)00718-X

separations into microfabricated systems, the difficulties lie in retaining packing material and maintaining packing homogeneity across the column on a chip [10].

Two alternatives to packed columns in microfluidic devices have been reported. Ramsey and co-workers [11,12] have fabricated a spiral shaped glass channel, coated with a C_{18} stationary phase, for performing electrokinetic reversed-phase chromatographic separation of trypsin-digested peptides. The stationary phase was directly attached to the silanol-rich glass walls. A second alternative was to mimic the packed bed by etching an array of supported particles into a quartz substrate [13,14]. Columns based on an array of collocate monolith support structures were employed for peptide separation using the capillary electrochromatography mode.

Currently, the majority of microfluidic devices are made from glass or silicon. Plastics, on the other hand, are inexpensive and compatible with replication techniques such as casting, embossing, injection molding, and imprinting [15]. McCormick et al. [16] reported an injection-molding protocol for microfabricating separation devices. Locascio and our laboratory developed the silicon template imprinting technique without heating plastic substrates during the stamping process [17]. Several research groups, including Whitesides and co-workers [18–21], Biebuyck et al. [22,23], and Effenhauser et al. [24] have demonstrated the use of an elastameric polymer, poly(dimethylsiloxane) (PDMS) to form microchannels from photoresist images on a silicon template. Furthermore, a laser ablation method for creating plastic microchannels was presented by Roberts and co-workers [25].

A capillary molding technique [26], the use of fused-silica capillary as a template for the fabrication of a PDMS microchannel along with the fluid inlet/outlet, was developed in our previous studies for the construction of a μ-enzyme reactor. Based on the large surface area to volume ratio of porous membrane media, trypsin was immobilized onto a poly(vinylidene fluoride) (PVDF) membrane for achieving ultrahigh trypsin concentration in the μ-enzyme reactor. The resulting peptide mixtures were either directly analyzed using electrospray ionization mass spectrometry (ESI-MS), or further concentrated and resolved by transient isotachophoresis/zone electrophoresis prior to the mass spectrometric analysis.

On-line coupling of the μ-enzyme reactor with μ-membrane chromatography is developed in this study for performing trypsin digestion and peptide separation in an integrated platform. The membrane media sandwiched between the PDMS microchannels in μ-membrane chromatography are employed as the stationary phase for the separation of peptides based on their differences in hydrophobicity. The peptide separations are monitored using UV absorbance at 220 nm and ESI-MS for protein identification. By comparing with the results obtained from μ-LC, the efficacy and the potentials of μ-membrane chromatography in tryptic mapping are reported.

2. Experimental

2.1. Materials and chemicals

Bovine pancreatic trypsin and horse heart cytochrome c were purchased from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris) and ultrapure urea were obtained from Bio-Rad (Hercules, CA, USA) and ICN (Aurora, OH, USA), respectively. Acetic acid, acetonitrile, methanol, and trifluoroacetic acid were acquired from Fisher (Fair Lawn, NJ, USA). All solutions were prepared using water purified by a Nanopure II system (Branstead, Dubuque, IA, USA) and further filtered with a 0.22-m membrane (Millipore, Bedford, MA, USA).

2.2. Fabrication and assembly of μ-enzyme reactor

The procedures for construction and assembly of a μ-enzyme reactor were described in detail in our previous work [26]. Briefly, fused-silica capillaries with the dimensions of 50 μm I.D.×192 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA) were used as the template for the fabrication of PDMS microchannels. As shown in Fig. 1, a symmetrically configured membrane reactor consisted of two aluminum plates, two copolyester slides, one PVDF membrane, and two PDMS substrates containing

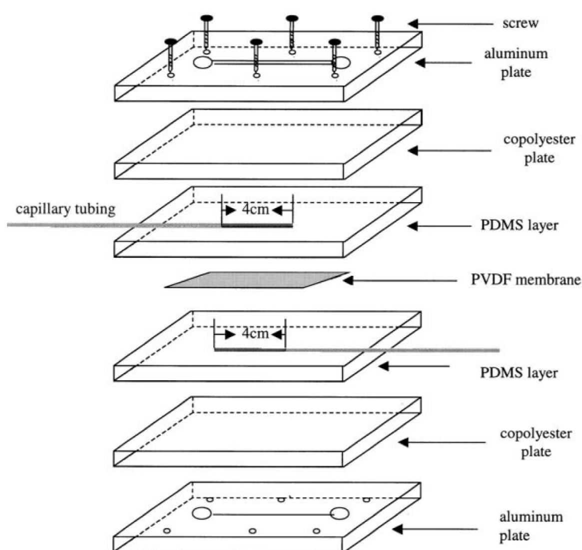

Fig. 1. Schematic of a μ-enzyme reactor assembly.

4-cm long microchannels along with the capillaries. The PVDF membranes with pore diameter of 0.1 μm (Immobilon-P^{SQ}) were acquired from Millipore. To assemble the μ-enzyme reactor, a PVDF membrane containing immobilized trypsin was first sandwiched between two PDMS substrates with the microchannels facing the membrane. The microchannels were aligned using the capillaries extending out of the PDMS substrates. Two copolyester slides were used to provide the additional support and clamped between two aluminum plates.

The capillary on the upper PDMS substrate (see Fig. 1) was connected to a Harvard Apparatus (Holliston, MA, USA) 22 syringe pump after the assembly of a μ-enzyme reactor. Horse heart cytochrome c was completely denatured in a solution containing 8 *M* urea and 10 m*M* Tris overnight at room temperature. Denatured cytochrome c was reconstituted in a solution of 10 m*M* Tris and 1 *M* urea using microcentrifuge ultrafiltration filters (regenerated cellulose membrane, nominal molecular mass cutoff of 5000, Millipore (Bedford, MA, USA). A 250-μl gas tight syringe (Hamilton, Reno, NV, USA) was filled with a solution containing denatured and reconstituted cytochrome c at a concentration of 4 mg/ml. The protein solution was delivered into the reactor at a flow-rate of 0.1 μl/min for performing trypsin digestion. The capillary on the lower PDMS substrate was connected to a 2-position/4-port sample injector/switching valve (Valco Instruments, Houston, TX, USA) (see Fig. 2) for peptide sample loading and on-line coupling with μ-membrane chromatography or μ-LC.

2.3. Coupling of μ-enzyme reactor with μ-membrane chromatography

The components and the procedures for the assembly of a μ-membrane chromatography system were similar to those of the μ-enzyme reactor, except the use of two layers of PVDF membrane and two PDMS substrates containing 0.5-cm long PDMS microchannels along with fused-silica capillaries (50 μm I.D.×100 μm O.D.). The capillary on the upper PDMS substrate and a Harvard Apparatus 22 syringe pump were connected to the column and the mobile phase ports of a Valco sample injector, respectively (see Fig. 2).

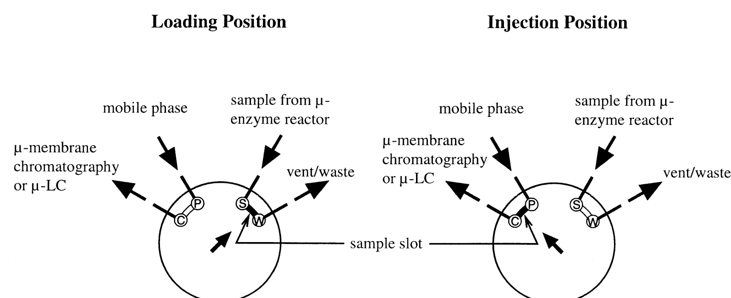

Fig. 2. On-line coupling of μ-enzyme reactor with μ-membrane chromatography or μ-LC through the use of a Valco sample injector. The injector contains four ports and can be switched between two positions for sample loading and injection.

The μ-membrane chromatography system was first flushed with a solution containing 20% (v/v) acetonitrile for 1 h and then equilibrated with deionized water for 2 h at a flow-rate of 0.2 μl/min. The cytochrome c digest from a μ-enzyme reactor was introduced into a 0.1-μl injection loop inside a Valco sample injector at a flow-rate of 0.1 μl/min. By switching from the loading to the injection positions, the mobile phase delivered by a syringe pump at a flow-rate of 0.2 μl/min performed sample injection and peptide separation through the μ-membrane chromatography system. Step elution was realized by changing the syringes that contained the solutions with various organic contents.

The capillary on the lower PDMS substrate was coupled to a Linear 100 multi-wavelength detector (Linear Instruments, Reno, NV, USA) at 220 nm or a microdialysis junction prior to a Perkin-Elmer Sciex (Foster City, CA, USA) API 150EX single quadrupole mass spectrometer. The microdialysis junction, which provided the necessary electrical connection for inducing the electrospray process, was constructed as described previously [27,28]. The eppendorf pipet tip housing the junction contained a solution of methanol–water–acetic acid (50:49:1, v/v/v) at pH 2.6. A platinum wire was inserted in the microdialysis junction and connected to a CZE 1000R high-voltage power supply (Spellman High-Voltage Electronics, Plainview, NY, USA). An electrospray voltage of 3.0 kV was employed for generating a stable spray of resolved peptides through a 2-cm-long ESI emitter capillary in the microdialysis junction. Peptides were measured by scanning from m/z 200 to m/z 1800 at a scan rate of 2 s/scan.

2.4. Coupling of μ-enzyme reactor with μ-LC

A 1-mm I.D.×15 cm reversed-phase C_{18} column (Phenomenex, Torrance, CA, USA) and an ABI 140C μ-LC pump (Applied Biosystems, Foster City, CA, USA) were connected to the column and the mobile phase ports of a Valco sample injector. Cytochrome c peptides from a μ-enzyme reactor were introduced into a 0.5-μl injection loop inside a Valco sample injector at a flow-rate of 0.1 μl/min. The mobile phase was delivered by a μ-LC pump at a flow-rate of 40 μl/min.

Solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B was 0.1% TFA in acetonitrile. Separation of the cytochrome c digest was effected with a linear gradient of 10–40% acetonitrile in 15 min. The acetonitrile content was kept at 40% for the remaining peptide separation. The column outlet was connected to a fused-silica capillary with the dimensions of 100 μm I.D.×200 μm O.D. and coupled with a Linear 100 multi-wavelength detector at 220 nm. Data collection was performed using a HP 35900D analog-to-digital interface board with HP G1250C General Purpose Chemstation Software (Hewlett-Packard, Fullerton, CA, USA).

3. Results and discussion

High-performance membrane chromatography (HPMC) has been successfully employed for the purification and separation of biological molecules [29–34]. In HPMC, layers of finely controlled and organized, microporous membrane are used as the stationary phase, and the functional ligands can be attached to the outer and inner surface areas of the membrane. The membranes as the porous separation media can be in the forms of a compact and porous disk, a hollow fiber, and a rod. By comparing with high-performance liquid chromatography, HPMC exhibits the advantages of low hydrodynamic pressure drop, rapid analysis, and less susceptibility to fouling and clogging. The use of various chromatography modes in HPMC, including affinity, ion-exchange, hydrophobic interaction, and reversed-phase, is dependent on the chemical modification of membranes.

In this study, the miniaturized HPMC system was developed by sandwiching PVDF membranes between two PDMS substrates containing microchannels and capillaries. This PVDF membrane (Immobilon-P^{SQ}, pore diameter of 0.10 μm) is naturally hydrophobic and is designed for Western transfers and protein sequencing procedures. The porous structure of this PVDF membrane provides a large internal surface area (200 cm^2/cm^2 of front surface) for peptide interaction.

Furthermore, the μ-membrane chromatography device was coupled to a μ-enzyme reactor for

performing on-line protein digestion and peptide separation. Various proteins and enzymes have been immobilized on the compact and porous membrane media for performing affinity chromatography and enzymatic reactions [35,36]. As discussed in our previous studies [26], the extent of protein digestion in a μ-enzyme reactor can be directly controlled by the residence time of protein analytes inside the trypsin-immobilized membrane, the reaction temperature, the protein concentration, and the membrane pore diameter. The residence time of protein analytes, in turn, is a function of membrane thickness, microchannel dimensions, and protein infusion rate. By applying the same experimental conditions as those reported previously [26], complete digestion of cytochrome c was anticipated and the resulting cytochrome c peptides were directly introduced into a μ-membrane chromatography system using a Valco sample injector.

As shown in Fig. 3a, cytochrome c peptides were eluted by a step-wise mobile phase gradient of 0–40% in 80 min and were detected by UV absorbance at 220 nm. Approximately 30 pmol of digested cytochrome c sample from the μ-enzyme reactor was injected for the separation. Theoretically, there should be 21 peptides in the cytochrome c digest based on the specificity of trypsin and the sequence of cytochrome c. Less than the theoretical number of peptides is frequently seen and reported for tryptic digests for several reasons. One is that very small, hydrophilic peptides elute from a reversed-phase column unretained. Another is that similar peptides co-elute during the separation.

By comparing with the separation results obtained from μ-LC (see Fig. 3b), the number of peaks in the two chromatograms is roughly the same. However, there are differences in the experimental conditions, detection sensitivities, and elution times that should be noted. One was that approximately 150 pmol of cytochrome c digest from the μ-enzyme reactor was injected for the separation in μ-LC. The sample consumption was five times of that utilized in μ-membrane chromatography. Another difference was the use of capillary window with the dimensions of 100 μm I.D.×200 μm O.D. for UV detection. By taking into account the difference in optical path length for absorbance detection, the concentration of eluted cytochrome c peptides from μ-membrane chromatography was at least twice than those measured from μ-LC.

The void volume inside two layers of PVDF membrane exposed to analyte molecules was estimated to be 80 nl. This estimation was based on a void volume of 0.8 μl per cm^2 of frontal surface area reported by Millipore. The total solution volume of 0.5-cm long microchannels in the upper and lower PDMS substrates was calculated to be 80 nl. In fact, most of the dead volume in μ-membrane chromatography was contributed by the capillary connections. At a flow-rate of 0.2 μl/min, the total void volume

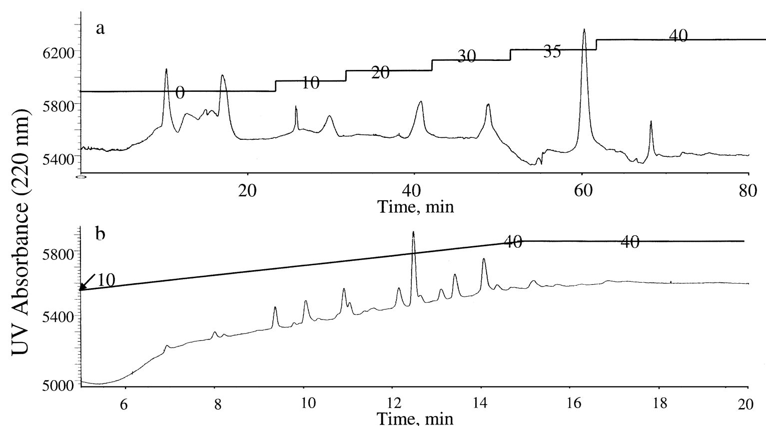

Fig. 3. Comparison of cytochrome c peptide separations achieved by (a) μ-membrane chromatography and (b) μ-LC with UV detection at 220 nm. The upper trace in each chromatogram represents the corresponding elution gradient during the separation.

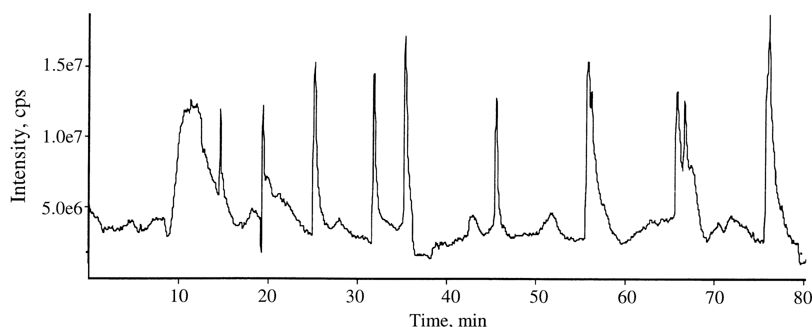

Fig. 4. Total ion current monitoring of cytochrome c peptides eluted from μ-membrane chromatography using the positive ESI mode.

in the system was measured as 1.60 μl. Significant reduction in the elution times of cytochrome c peptides can be achieved especially by minimizing the dead volume prior to and after the chromatographic separation. For example, on-channel laser-induced fluorescence detection of labeled peptides not only shortens the analysis time, but also improves detection sensitivity in our future studies.

Instead of using UV detection, the low flow-rate of μ-membrane chromatography makes it possible to directly couple with a microdialysis junction containing a nanospray emitter capillary. On-line ESI-MS analysis of separated cytochrome c peptides from μ-membrane chromatography is shown in Fig. 4. By comparing with the results shown in Fig. 3a, the increase in the migration time of cytochrome c peptides was attributed to the increase in the dead volume in association with the microdialysis junction and additional capillary connection. All the cytochrome c peptides in the regenerated total ion chromatogram were directly identified on the basis of mass spectra taken from the average scans under the peaks. The mass spectra of peptides taken from the average scans under the peaks with the elution times

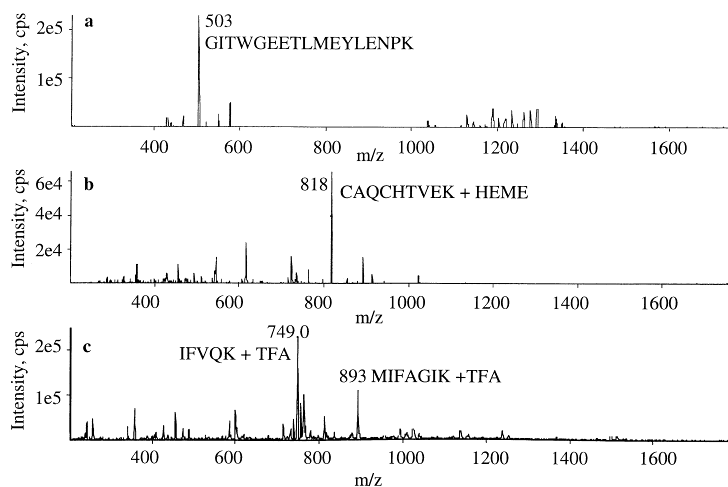

Fig. 5. Positive ESI mass spectra taken from the average scans under the peaks with the elution times of (a) 76.1 min, (b) 52.0 min, and (c) 35.5 min in Fig. 4.

of 76.1 min, 52.0 min, and 35.5 min are shown in Fig. 5. The amino acid sequence of each peptide was obtained by searching the database using the mass as the constraint.

4. Conclusion

On-line coupling of a μ-enzyme reactor with μ-membrane chromatography is developed and demonstrated as an integrated microanalytical tool enabling rapid protein digestion, high resolution peptide separation, and sensitive protein identification using ESI-MS. The combination of chromatographic separation with ESI-MS contributes to further enhancement in the dynamic range and the detection sensitivity for the analysis of a protein digest. Such enhancement is particularly attractive for the analysis of complex protein mixtures with a significant difference in their individual concentrations.

To further enhance the separation performance of a protein digest in μ-membrane chromatography, a linear elution gradient (instead of the step-wise gradient) will be investigated in our future studies. A linear elution gradient at a flow-rate of 10–200 nl/min can be generated from a regular μ-LC pump using a precolumn splitter together with a flow restrictor [37]. The capacity factors, k', measured from μ-membrane chromatography are smaller than those obtained from μ-LC using a reversed-phase C_{18} column. For example, the capacity factor for the peptide fragment CAQCHTVEK+HEME (see Fig. 5) was calculated to be around 5.5 in μ-membrane chromatography and 8.0 in μ-LC. By changing to a more hydrophobic membrane media or chemically modifying the membranes [30,38], stronger retention and better resolution of protein digest may be obtained in μ-membrane chromatography.

Acknowledgements

Support for this work by the Analytical Chemistry Division of the National Institute of Standards and Technology and National Cancer Institute are gratefully acknowledged.

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