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Capillary high-performance liquid chromatography–electrospray ionization mass spectrometry using monolithic columns and carbon fiber electrospray ionization emitters

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Abstract

Monolithic columns having long hydrocarbon chains were prepared by in-situ polymerization in capillary fused silica tubing. The capillary columns were coupled with a newly developed carbon fiber electrospray ionization (ESI) emitter for proteomic analysis using sheathless capillary HPLC–ESI mass spectrometry (MS). The sample loading capacity and chromatographic performance of the styrene-based monolithic column, which was prepared by photo-polymerization of octylstyrene (OS) and divinylbenzene (DVB) were compared with that of the methacrylate-based monolithic column composed of lauryl methacrylate (LMA) and ethylene dimethacrylate (EDMA). The sample loading ability of tryptic digested protein in poly-OS (POS)–DVB column was higher than that of poly-LMA (PLMA)–EDMA column, possibly due to the irregular and rugluous surface offering a greater surface area of POS–DVB stationary phase. The POS–DVB column also provided better separation efficiency in the separation of high concentration (10 μg) of tryptic digested albumin bovine serum (BSA). Due to the successful interface of a highly efficient monolithic column and a stable, durable carbon fiber emitter, low femtomole levels of peptides were successfully separated and identified in the presence of large amounts of tryptic digested protein.

Keywords: Electrospray ionization emitters; Monolithic columns; Instrumentation; Peptides

1. Introduction

Identification and quantification of the expressed protein components of biological origin require sensitive and high-throughput analytical technologies. Two-dimensional gel electrophoresis (2DE) is a powerful technique for protein separation [1,2]. However, it has several limitations, such as long separation time, limited loading capacity, difficulty in automation, and a low resolving power for small proteins. Liquid chromatographic techniques are faster, easier to automate, and couple more readily to mass spectrometry than 2DE. To supplement the resolving ability of liquid chromatographic separation systems, a powerful alternative technique, multidimensional chromatographic analysis method, has been realized [3–6].

Another important prerequisite for proteomic analysis is the ability to handle very small amounts of biological material and to have sufficient dynamic range to facilitate detection of low-level proteins with high sensitivity in the presence of high-abundance proteins. Capillary liquid chromatography (LC) coupled with electrospray ionization mass spectrometry (ESI-MS) has been an important tool for the analysis of complex peptide mixtures from proteolytic digests [7,8]. The replacement of conventional analytical scale chromatography (1–2 mm i.d. columns) with capillary LC (50–100 μm i.d. capillary columns) brings some benefits to the separation process, such as higher sensitivity, and lower sample and mobile-phase consumption.

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Recently, the preparation of organic polymer-based monolithic stationary phases in capillary columns has attracted increasing attention in liquid chromatography [9–13]. The key advantages provided by polymer-based monolithic columns include the easy preparation, high performance, modifiable properties such as porosity, surface area and functionality, chemical stability over the pH range of 1–14, and absence of frits to retain the packed bed compared to a conventional columns packed with particles for the separation of biopolymers. The polymeric monolithic columns include styrene-based, acrylate- or methacrylate-based, and acryamide-based polymers. Polymeric monolithic columns are usually prepared by UV or thermally induced in-situ polymerization of a mixture of suitable monomers and porogenic solvents within a capillary that acts as a mold. The porous structure in the monolith is achieved as a result of the phase separation of the solid polymer from the porogenic solvents during the course of polymerization. Size and morphology of the pores strongly depend on several factors, including polymerization kinetics and solvency of the porogenic solvents for the resulting polymer and their proportion in the polymerization mixture. Microporogenic solvents have good solvency for the polymer, which results in the formation of small pores, whereas macroporogenic solvents exhibit only poor solvating capabilities for the polymer and contribute to preparation of macro-sized pores [14,15].

Polymeric monolithic columns have been applied to the separation of biomolecules such as proteins, peptides [16–25], and polynucleotides [24–27]. Especially, poly (styrene–divinylbenzene) (PS–DVB) particles and monolith have been widely used for reversed-phase liquid chromatographic stationary phase due to its hydrophobic surface [21–28]. However, for the separation and identification of smaller peptides, it has been reported that modifications of PS–DVB monolith by surface alkylation provided better resolution of peptides [29,30].

Recently, research in ESI-MS has focused on the reduction of sample consumption and increased sensitivity in the analysis. These requirements have led to low-flow rate emitter tips such as nanospray emitters [31]. The reduction of the flow rate produces smaller droplets having a high surface-to-volume ratio. This gives advantages for ESI-MS, such as increased sensitivity. As a consequence, much effort has been put into the production of high-performing, physically and chemically stable nanospray needles and low-flow ESI emitters [32–34]. However, the susceptibility to clogging of the tip during the operation procedure limits the application of tapered tips with small inside diameters. We have recently developed a novel carbon fiber emitter which is designed for highly sensitive, stable, and durable electrospray ionization with a minimization of the clogging problem [35].

In this study, we report the preparation of styrene- and methacrylate-based monolithic capillary columns coupled with a carbon fiber emitter. To increase the hydrophobicity and loading capacity of the stationary phase, monomers with long hydrocarbon chains, e.g. octylstyrene and lauryl methacrylate, were used for in-situ preparation of monolithic columns. The column performances of sample loading capacity and separation efficiency of tryptic digested proteins were evaluated and compared. The applicability of the interface of the monolithic column with the carbon fiber emitter in a model proteomic study was demonstrated by the separation and identification of a low femtomole level spiked peptide in the presence of large amounts of a tryptic digested protein.

2. Experimental

2.1. Materials

Fused-silica capillary tubing of 100 μm i.d. × 375 μm o.d. with UV-transparent fluorinated hydrocarbon polymer coating and 75 μm i.d. × 375 μm o.d. with polyimide coating were obtained from Polymicro Technologies (Phoenix, AZ, USA). 3-(Trimethoxylsilyl)propyl methacrylate, lauryl methacrylate (LMA), ethylene glycol dimethacrylate (EDMA), divinylbenzene (DVB; mixture of isomers including 3- and 4-ethylvinylbenzene), 1-propanol, 1,4-butanediol, 1-decanol, tetrahydrofuran (THF), 2,2-dimethoxy-2-phenylacetophenone (DPA), 2,2′-azobisisobutyronitrile (AIBN), dithiothreitol (DTT), and iodoacetamide were purchased from Aldrich (Milwaukee, WI, USA). 4-Octylstyrene (OS) was obtained Tokyo Kasei (Tokyo, Japan). Trifluoroacetic acid (TFA), trichloroacetic acid (TCA), acetonitrile, methanol, ammonium hydrogen carbonate and basic alumina were obtained from Fisher Scientific (Pittsburg, PA, USA). Gly–Gly–Gly, albumin bovine serum (BSA), myoglobin (horse heart) and trypsin (proteomics grade) were purchased from Sigma (St. Louis, MO, USA). LMA, EDMA, DVB, and OS were prepared by passing through basic alumina to remove the inhibitors. AIBN was purified by recrystallization from methanol. The other materials were used without further purification.

2.2. Monolithic column preparation

The vinylization of the capillary tubing was carried out as previously reported [36]. Briefly, fused-silica capillary tubing (100 μm i.d. × 375 μm o.d.) with UV-transparent fluorinated hydrocarbon polymer coating was rinsed with acetone and water using a syringe, activated with 0.2 M sodium hydroxide for 30 min, washed with water followed by 0.2 M HCl for 30 min, then washed with water and acetone, and dried by passing through the helium. The capillary was filled with a 30% (v/v) 3-(trimethoxylsilyl)propyl methacrylate in acetone, sealed, and left to react for 24 h at room temperature. The modified capillary was washed with acetone and dried.

Two kinds of monolithic columns, poly(octylstyren–co-divinylbenzene) (POS–DVB) column and poly(lauryl methacrylate–co-ethylene glycol dimethacrylate) (PLMA–EDMA) column, were prepared by polymerization of monomers, porogenic solvents, and a polymerization

initiator. For the preparation of the POS–DVB column, the polymerization mixture consisted of a monomer mixture (containing 50 μl of OS and 50 μl of DVB), porogenic solvents (mixed from 194 μl of decanol and 30 μl of THF) and 1 mg of AIBN as a polymerization initiator was used. The PLMA/EDMA column was prepared using a monomer mixture consisting of 180 mg LMA and 120 mg EDMA, porogenic solvents of 204 mg 1-propanol and 61 mg 1,4-butanediol, and 3 mg of DPA as an initiator. The polymerization mixture was mixed ultrasonically into a homogenous solution and purged with helium for 3 min. A 30 cm-long capillary was attached to the syringe, filled with the mixture, and sealed with PTFE tubings. Control of location of the monolith was facilitated by the use of aluminum foil mask. Unpolymerized mixture could be readily flushed from the capillary after completion of the polymerization procedure. The length of the unmasked area was kept constant at 10 cm. Polymerization was initiated by placing the capillary in a box equipped with five 8-W UV lamps and irradiating for 18 h (POS–DVB column) or 20 min (PLMA–EDMA column) at 25 °C. After the polymerization was completed, the monolithic column was washed with methanol for 12 h using a HPLC pump to remove unreacted monomers and porogenic solvents.

2.3. Preparation of carbon fiber emitter

Carbon fiber emitters were constructed from a length of fused-silica tubing with polyimide coating. In the emitter terminus of the tubing, a 5 mm length of carbon fiber (30 μm o.d., World Precision Instruments, Sarasota, FL, USA) was inserted with the use of carbon ink adhesive (Conductive Compounds, Londonderry, NH, USA). The carbon fiber was allowed to protrude ∼0.2 mm from the tubing terminus. The protruding carbon fiber was etched to a pointed shape by application of a discharge from a Tesla coil (BD-20, Electro-Technic Products, Chicago, IL, USA). The pointed carbon fiber protruded ∼20–30 μm from the tubing terminus. The assembly was sputter-coated with a layer of gold utilizing a scanning electron microscopy (SEM) coating unit (E5150, Polaron Equipment Ltd., Doylestown, PA, USA). The gold was then coated with a thin layer of Krytox 1625 perfluoralkyl ether oil (Dupont, Wilmington, DE, USA).

2.4. Sample preparation

One milligram of BSA was dissolved in 1.0 ml ammonium hydrogen carbonate solution (50 mM), reduced with 3 mM of DTT at 56 °C for 1 h, and alkylated with 15 mM of iodoacetamide in the dark at room temperature for 45 min. The alkylated BSA was precipitated with TCA to 10% at 0 °C for 30 min, centrifuged, and the supernatant was removed. The pellet was washed twice with water and dissolved with 1.0 ml ammonium bicarbonate solution. Twenty micrograms of trypsin (1:50) was added to the BSA solution and allowed to incubate at 37 °C overnight. The digest mixture was acidified with TFA to 1%, lyophilized to dryness, and dissolved in 100 μl water to make 10 μg/μl tryptic digested BSA solution.

Myoglobin (0.5 mg) was dissolved in 1.0 ml ammonium hydrogen carbonate solution (50 mM) and denatured in the boiling water for 30 min. Ten micrograms of trypsin (1:50) was added to the myoglobin solution and allowed to incubate at 37 °C overnight. The digest was acidified with TFA to 1%, lyophilized to dryness, dissolved in 1.0 ml water, and diluted with water to make 100 fmol/μl.

2.5. Instrumentation

In the HPLC–ESI-MS system for evaluating monolithic columns and the carbon fiber emitter, a solvent delivery system (140B, Applied Biosystems, Foster City, CA, USA) was interfaced with a quadrupole ion trap mass spectrometer (LCQ Classic, Thermo Finnigan, San Jose, CA, USA). A PEEK tee (Upchurch Scientific, Oak Harbor, WA, USA) was connected after the solvent delivery system and before an injection valve (Upchurch Scientific) with a 1.0 μl sample loop, which typically achieved a 1–100 split of the flow to the column while the remainder was directed to waste. The mobile-phase flow rate before the split was, unless otherwise noted, maintained at 30 μl/min, while the postsplit flow rate for the column was maintained at 300 nl/min. The monolithic column was directly connected to the carbon fiber emitter by means of a microtight union (Upchurch Scientific). For MS analysis, the spray voltage was adjusted to 2.5 kV and an inlet capillary temperature of 200 °C was used for all experiments. The instrument was operated in a data-dependent tandem MS mode in which each full-scan mass spectrum was followed by a tandem MS scan of the most intense ion observed in the previous scan. Dynamic exclusion was performed with a repeat count of 3, a repeat duration of 0.5 min, and a 3-min exclusion duration window. Normalized collision energy was set to 35%. The MS was controlled and total ion chromatograms and mass spectra were recorded using ThermoFinnigan Xcalibur software (San Jose, CA, USA).

2.6. Loading capacity measurement

Monolithic columns were preconditioned using 70% acetonitrile in 0.15% aqueous TFA solution followed by 5% acetonitrile in 0.15% aqueous TFA solution. A 500 μl injection loop in an injection valve (8125, Rheodyne, Cotati, CA, USA) was filled with 1 μg/μl of digested BSA solution in 5% acetonitrile in 0.15% aqueous TFA solution and this solution was pumped into the monolithic column at a flow rate of 300 nl/min. The effluent was monitored by the selected ion monitoring (SIM) for the doubly charged peptide ion at m/z 821. To determine the time required for the sample solution to flow through columns, 0.1 μg/μl of Gly–Gly–Gly in 70% acetonitrile in 0.15% aqueous TFA solution was used as an unretained marker and monitored by the SIM at m/z 189. The loading capacities were evaluated based on the retention volume and the concentration of digested BSA.

2.7. Separation efficiency and sensitivity tests

The tryptic digested proteins were separated using a gradient HPLC system with a monolithic capillary column. The flow rate was maintained at 300 nl/min after the flow split. The gradient was started at 5% B for 5 min, and ramped to 100% B in 60 min, where mobile phase A was 3% acetonitrile in 0.15% aqueous TFA and mobile phase B was 70% acetonitrile in 0.15% aqueous TFA. For the separation efficiency test, 1 μl of tryptic digested BSA (10 μg/μl) was injected from the injection valve onto a monolithic column. Peptide ions were detected in a full MS scan from 400 to 2000 *m*/*z* followed by data-dependent MS/MS scans between 400 and 2000 *m*/*z*. The sensitivity test was carried out by adding specific amounts of digested myoglobin into digested BSA solution. One microlitre of tryptic digested BSA (10 μg/μl) containing digested myoglobin was injected onto a column. A target peptide (HGTVVLTALGGILK, monoisotropic *m*/*z* of 690.3 for doubly charged ion) from myoglobin was detected in a MS scan from 690 to 691 *m*/*z* followed by a data-dependent MS–MS scan (400–2000 *m*/*z*).

3. Results and discussion

3.1. Preparation of monolithic columns

In this study, two kinds of monolithic columns, POS–DVB and PLMA–EDMA, were prepared and their chromatographic characteristics were compared. Covalent bonding between a polymer monolith and the inner wall of a fused-silica capillary is necessary for mechanical stability and to prevent a gap at the inner wall of capillary that degrades chromatographic resolution. We therefore functionalized the capillary inner wall using 3-(trimethoxysilyl)propyl methacrylate to enable covalent attachment of the monolith to the wall.

HPLC columns based on PS–DVB have been used for the separation of various polymers with high efficiency due to the hydrophobic surface of PS–DVB [21–30]. However, the reversed-phase LC chromatographic resolution on the unfunctionalized PS–DVB particles was poor for the separation of smaller peptides [37,38]. It has been shown that an alkylation of PS–DVB particles, to graft octadecyl chains on their surfaces, is necessary to achieve good resolution for peptides [29]. Huang et al. [30] has also shown that surface octadecylation of PS–DVB monolith via a Friedel-Crafts alkylation reaction provided an improved chromatographic separation of peptides compared to an unmodified monolith PS–DVB column.

In the procedure described here, OS was used for the in-situ preparation of monolithic column without any additional surface reaction or modification of the polymer. For the polymerization of OS and DVB, decanol and THF were used as macro- and meso-porogenic solvents [23–26]. OS was readily dissolved in the porogenic solvents without any precipitation. Photopolymerization was accomplished with the use of AIBN as an initiator. A polymerization reaction using a DPA as an initiator, which is used for the polymerization of the LMA–EDMA monolithic column, was not successful even after 24 h of reaction time.

A methacrylate ester-based monolithic column including LMA has shown better separation performance and an increased capacity factor in the reverse-phased hydrophobic amino acid separation compared with that of butyl methacrylate monolithic columns because of stronger hydrophobicity of lauryl material [18]. Separation of small peptides has also been successfully realized with high column efficiency in PLMA–EDMA monolith columns using a capillary electrochromatography method [19].

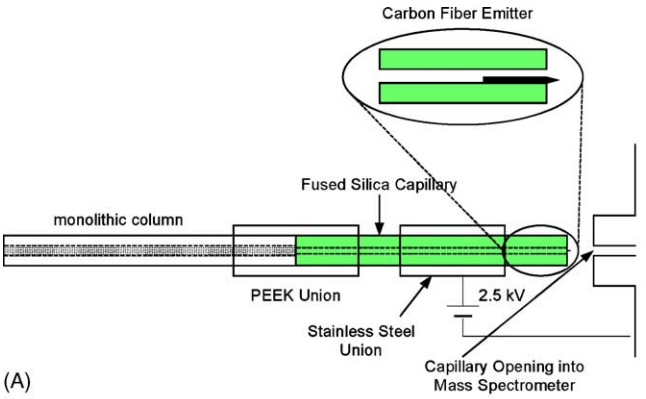
The PLMA–EDMA monolithic column was prepared by a method similar to that reported by Wu et al. [19]. However, to increase the surface area by decreasing the monolith pore size, the content of the porogenic solvent in the polymerization mixture was set to 47% and the proportion of 1-propanol in the porogenic solvent was increased to 77%. It has been reported that use of a higher percentage of 1-propanol promotes smaller pore diameters and leads to construction of a monolith with higher column efficiency [39].

3.2. Carbon fiber emitter

The carbon fiber emitter is designed for highly sensitive, stable, and durable electrospray ionization without the clogging problem. The simplified schematic for the electrospray interface of a monolithic column with a carbon fiber emitter and the image of electrospray ionization from a pointed carbon fiber emitter are shown in Fig. 1. The spray voltage is applied to a gold-coated carbon fiber emitter through the stainless steel union. Because the surface of the end of the capillary, covered by perfluoroalkyl ether film, is hydrophobic, we observed that the diameter of a Taylor cone was almost equal to the internal diameter of the fused silica capillary (Fig. 1). A stable, symmetric Taylor cone can be generated in the spray voltage of 1.5–4.5 kV and at the flow rate range of 0.05–5.0 μl/min [35]. The gap between the carbon fiber and the inner wall of the capillary is wider than 20 μm, which avoids the risk of clogging and becomes reliable electrospray emitter for highly stable and sensitive electrospray ionization. The electrospray ionization from carbon fiber was very stable and could last 15–20 h continuously without a significant signal intensity change, indicating that the carbon fiber emitter is suitable for long-term electrospray ionization purposes such as interfacing with capillary HPLC. The carbon fiber emitter also provided two-fold greater sensitivity compared to a commercial nanoemitter (New Objective PicoTip, 8 μm orifice, Woburn, MA, USA), therefore it has been used to detect peptide signals and collect the data dependent sequencing information at low femtomole levels of peptides [35].

3.3. Sample loading capacity

The loading capacities of two monolithic columns were evaluated based on breakthrough curves [40,41]. A high



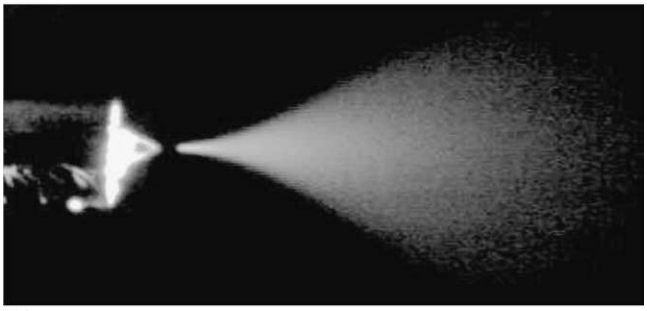


Fig. 1. (A) Schematic illustration of the interface of capillary HPLC including a monolithic column and a carbon fiber emitter to ESI-MS. (B) Image of electrospray ionization from a pointed carbon fiber emitter.

loading capacity of a stationary phase enables large scale separations. A small peptide, Gly–Gly–Gly, in 70% acetonitrile was used as an unretained marker to measure the delay time of analytes eluting through the column. A mixture of peptides resulting from the tryptic digest of BSA was used as the test analyte. The tryptic BSA peptide (KVPQVSTPTLVEVSR, monoisotropic *m*/*z* of 821 for doubly charged ion) was monitored for this study.

For the determination of void volumes in both monolithic columns, the test solutions (500 μl) of Gly–Gly–Gly were loaded onto the columns in a flow of 70% acetonitrile, and the effluents were monitored by SIM, respectively. The Gly–Gly–Gly reached its ion current maximum rapidly because it was not retained in the column (Fig. 2, curve A). The time delays to reach 10% of the ion current maximum in the PLMA–EDMA column and the POS–DVB column were 15.7 min and 15.5 min, respectively. The almost same time delays indicate that the marker, Gly–Gly–Gly in 70% acetonitrile, is not retained and is eluted with same speeds in both columns.

However, the chosen test peptide from digested BSA was retained and saturated the monolithic columns in a flow of 5% acetonitrile. Upon saturation, excess, unretained peptide passed through the column, as indicated by the ion current maximum (Fig. 2, curves B and C). Based on the time delay to reach 10% of the ion current maximum, the flow rate, and the concentration of digested BSA, the loading capacities

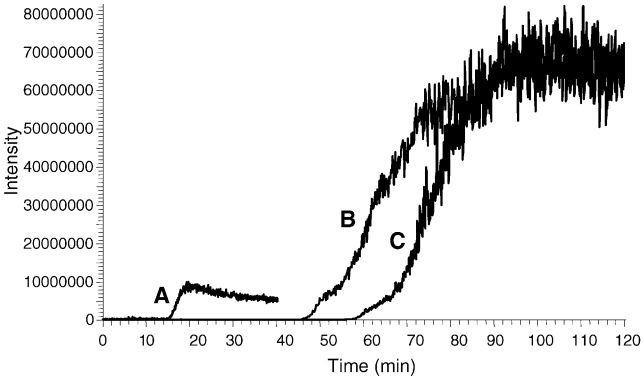


Fig. 2. Breakthrough curves for sample loading capacity evaluation in monolithic columns: flow rate, 300 nl/min. (A) 0.1 μg/μl of Gly–Gly–Gly in 70% acetonitrile in 0.15% aqueous TFA solution passed through the POS–DVB monolithic column and monitored by the SIM at *m*/*z* 189; 1.0 μg/μl of digested BSA solution in 5% acetonitrile in 0.15% aqueous TFA solution passed through (B) PLMA–EDMA and (C) POS–DVB monolithic column and monitored by the SIM at *m*/*z* 821 for the detection of a test peptide (KVPQVSTPTLVEVSR) of tryptic digested BSA.

were evaluated to be 11.3 μg (PLMA–EDMA) and 15.5 μg (POS–DVB).

However, the loading capacity of PLMA–EDMA column was found to be lower than that of POS–DVB column in spite of the longer alkyl chain of PLMA–EDMA column. In order to understand the differences in loading capacity in the two columns, the polymer morphology in the capillaries was examined by scanning electron microscope (SEM). The SEM micrographs showed significant difference in morphology between the POS–DVB and PLMA–EDMA columns (Fig. 3). While the surface of the POS–DVB monolith was rather irregular and rugulose (Fig. 3A), the surface of the PLMA–EDMA monolith was round and smooth (Fig. 3B). During polymerization of POS–DVB, small primary particles seemed to coagulate to form the porous monolith, which offers a greater surface area and smaller pore diameter than that of the smooth spherical particle shown in PLMA–EDMA columns. The SEM micrographs also showed that the monolith of POS–DVB was composed of linked nodules that are less than 1 μm in diameter. These nodules are much smaller than those of the PLMA–EDMA monolith.

It is likely that, the irregular and rugulose surface and smaller nodules contributed to the higher loading capacity of the monolithic column. This finding correlates with an earlier observation made with PS–DVB and norbornene-based monoliths, where a rugulose surface facilitated more efficient separation of proteins or nucleic acid [26,42].

3.4. Separation efficiency test

To characterize the interface of the monolithic capillary column and the carbon fiber emitter for the analysis of peptide mixtures, the separation efficiency in POS–DVB and PLMA–EDMA monolithic columns was compared by analyzing a peptide mixture from 10 μg of trypsin digested BSA.

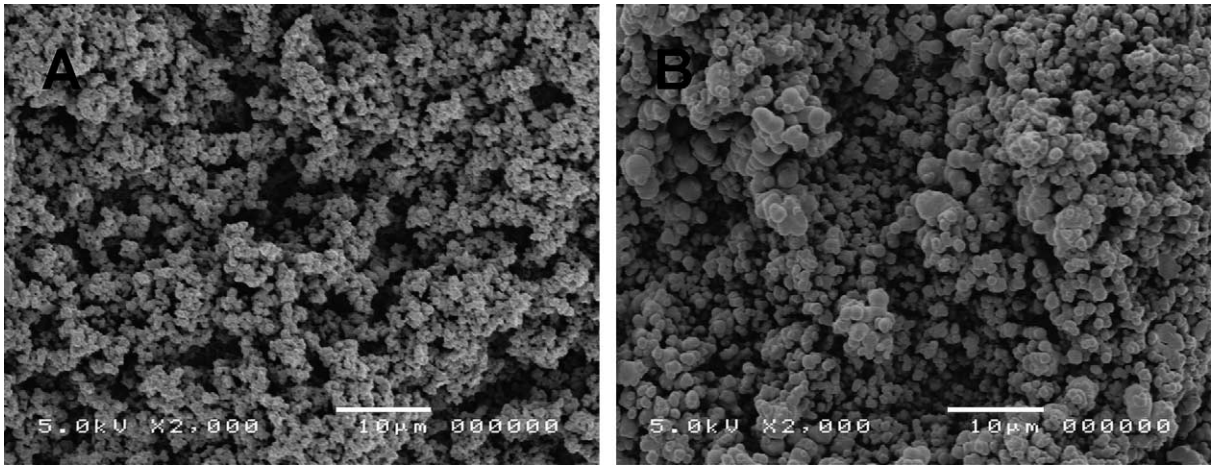


Fig. 3. SEM images of (A) POS–DVB and (B) PLMA–EDMA monolithic columns.

Figs. 4 and 5 show base peaks and select extracted ion chromatograms, corresponding to individual peptides with relatively high intensities. The void volume in the carbon fiber emitter did not substantially contribute to extracolumn band broadening, because samples were injected in relatively weak mobile phases to allow to stack at the head of the monolithic columns and because the 75 μm i.d. tubing at the outlet of the column would be expected to exhibit laminar flow and thus not result in a mixing dead volume. A stable baseline was achieved through the gradient elution. This stability indicated that the protruded carbon fiber promoted stability of the electrospray ionization during surface tension changes occurring during the ramping of organic solvent in the mobile phase.

In base peak chromatograms, the POS–DVB column (Fig. 4) shows better baseline separation of peptides than the PLMA–EDMA column (Fig. 5). The higher quality separation obtained with the POS–DVB column could be due to irregular and rugulous particle surface offering a larger contact area than that of smooth particles in PLMA–EDMA stationary phase and smaller pore diameters of POS–DVB stationary phase. In selected extracted ion chromatograms obtained from both columns, almost all peptides eluted to provide chromatographic peaks of good resolution and peak shape. Both columns can afford the ability to load the large mass of sample that is often desirable for proteomic LC–MS study.

To compare the column efficiency in both columns, peak widths at half-height and resolution values were evaluated. Table 1 shows the peak widths at half-height and resolution values that were measured from the extracted ion chromatograms (Figs. 4 and 5). The peak widths at half-height

Table 1
Identification of selected peptides from BSA tryptic digest, peak widths and resolution values of each peak on the POS–DVB and PLMA–EDMA monolithic columns

Peptide	Residues	Sequence	POS–DVB		PLMA–EDMA	
			$W_{1/2}$ (min)	R^a	$W_{1/2}$ (min)	R^a
1	490–495	TPVSEK	0.45	–	0.49	–
2	29–34	SEIAHR	0.45	0.76	0.51	1.60
3	101–105	VASLR	0.60	1.30	1.52	1.05
4	242–248	LSQKFPK	1.48	10.85	0.55	8.20
5	198–204	GACLLPK	0.94	2.14	0.78	2.69
6	249–256	AEFVEVTK	0.75	0.72	0.69	0.50
7	387–399	DDPHACYSTVFDK	0.61	4.06	0.49	3.18
8	402–412	HLVDEPQNLIK	0.49	0.57	0.50	0.31
9	161–167	YLYEIAR	0.58	0.66	0.66	0.20
10	437–451	KVPQVSTPTLVEVSR	0.59	0.93	0.65	1.05
11	66–75	LVNELTEFAK	0.57	1.70	0.68	1.63
12	508–523	RPCFSALTPDETYVPK	0.63	0.54	0.77	0.71
13	360–371	RHPEYAVSVLLR	0.56	1.71	0.89	0.38
14	89–100	SLHTLFGDELCK	0.84	0.44	0.95	0.51
15	421–433	LGEYGFQNALIVR	0.54	1.49	0.77	1.50
16	168–183	RHPYFYAPELLYYANK	0.63	3.90	0.85	2.82
17	469–482	MPCTEDYLSLILNR	0.49	1.55	0.74	0.90
18	45–65	GLVLIAFSQYLQQCPFDEHVK	0.58	4.06	0.78	3.97

[a] R is the resolution value calculated with the preceding peak.

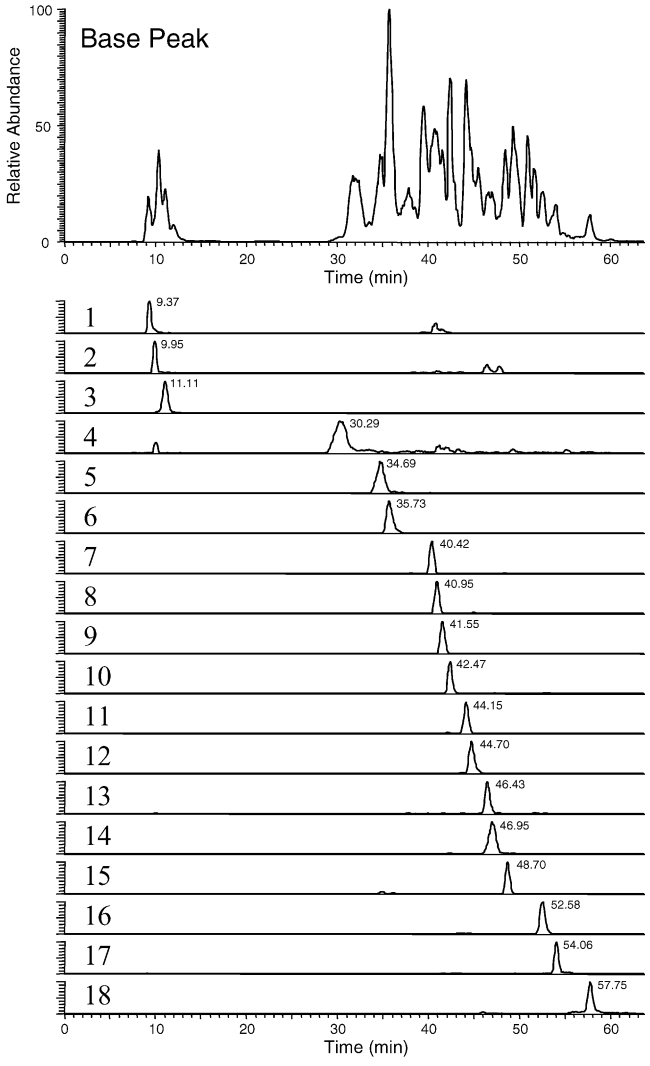


Fig. 4. Base peak chromatogram and extracted ion chromatograms from the LC–ESI-MS analysis of tryptic digested BSA on a POS–DVB monolithic column (100 μm i.d., 375 μm o.d., 100 mm long). Sample: 10 μg/μl of tryptic digested BSA. Mobile phase: A, 3% acetonitrile in 0.15% aqueous TFA solution; B, 70% acetonitrile in 0.15% aqueous TFA solution. Gradient elution program: 0 min → 5 min → 65 min, 3% → 3% → 100% B. Flow rate in column: 300 nl/min. Voltage for ESI with the carbon fiber emitter: +2.5 kV. MS detection: 400–2000 *m*/*z*.

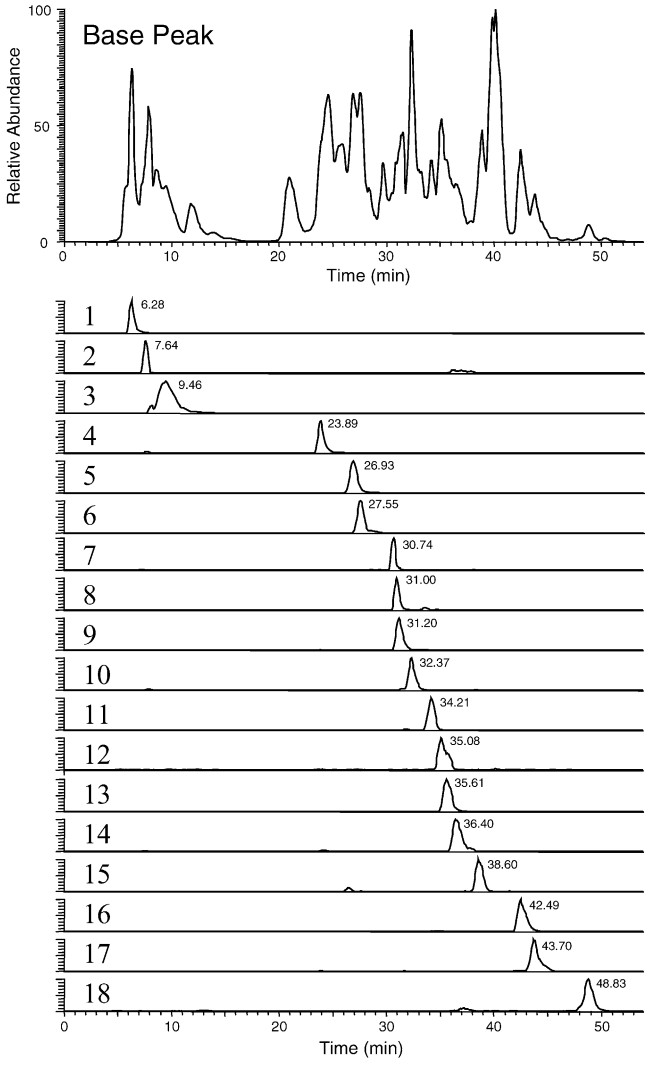


Fig. 5. Base peak chromatogram and extracted ion chromatograms from the LC–ESI-MS analysis of tryptic digested BSA on a PLMA–EDMA monolithic column (100 μm i.d., 375 μm o.d., 100 mm long). Sample: 10 μg/μl of tryptic digested BSA. Other conditions are the same as those in Fig. 4.

from most of the tryptic peptides (except peptides 4–7) on the POS–DVB column were narrower than those obtained from the PLMA–EDMA column. The resolutions ranged from 0.54 to 10.85 in the POS–DVB column, while those in the PLMA–EDMA column were 0.20–8.20. The resolutions of most peaks were better in the POS–DVB column compared to the PLMA–EDMA column. These results indicate that the POS–DVB column has superior separation efficiency, as compared to the PLMA–EDMA column. Therefore, comparisons of both loading capacity and column efficiency in the columns indicate that the POS–DVB column provides much better reversed-phase LC separation of peptides than the PLMA–EDMA column under the same gradient elution conditions.

3.5. *Sensitivity test*

In the course of separations of digests of protein mixtures, some peptides can be present in very large amounts and such high-abundance peptides can interfere with the detection of low-abundance peptides. Detection of peptides coming from low-abundance proteins requires a large dynamic range for the separation and detection process. A route to obtaining such large dynamic range is the combination of a high resolution separation with a sensitive detection system. To further evaluate the interface of our monolithic columns with the carbon fiber emitter for LC–ESI-MS and to explore their applications to proteomics, sensitivity tests for the detection of low abundance proteins in high concentration protein mixtures were carried out.

As a model peptide to detect a low abundance tryptic digest protein fragment in the presence of more abundant

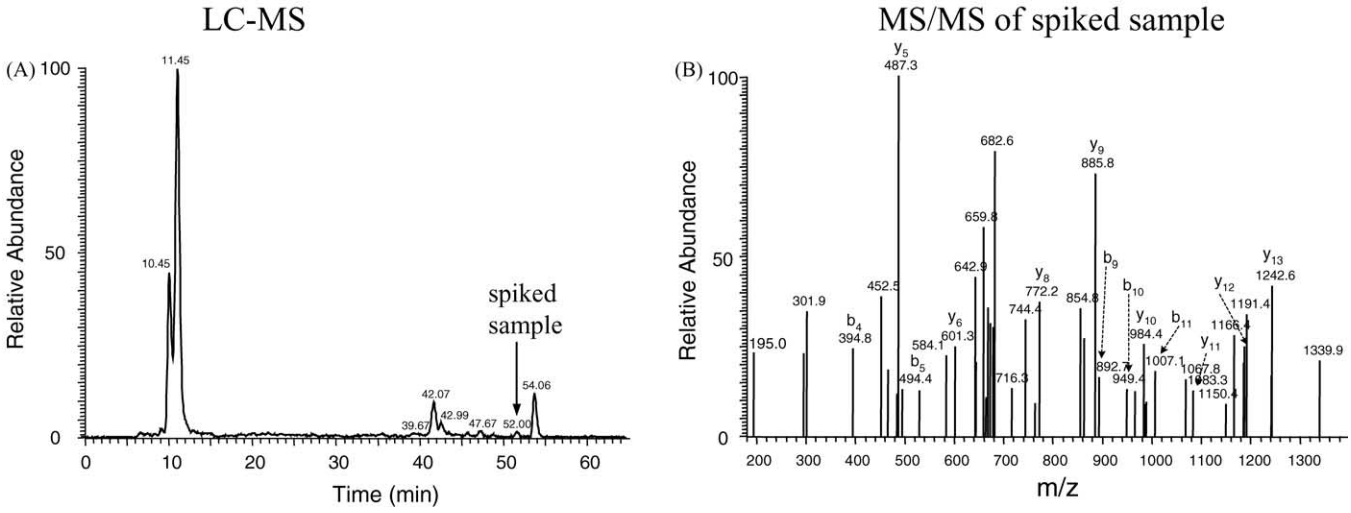


Fig. 6. LC–MS–MS identification of 500 amol/μl peptide from trypic digested myoglobin which was spiked in 10 μg/μl of digested BSA on a POS–DVB monolithic column (100 μm i.d., 375 μm o.d., 100 mm long). (A) The base peak chromatogram in the scan range of 690–691 *m/z*. (B) The MS–MS spectrum of the detected target peptide (HGTVVLTALGGILK) selected from digested myoglobin. Other conditions are the same as those in Fig. 4.

fragments, a peptide (HGTVVLTALGGILK (MH^+: 1379.7), doubly charged ion at *m/z* 690.3) from tryptic digested myoglobin was selected. Specific amounts of digested myoglobin were added to 10 μg/μl of digested BSA, separated and analyzed on an LC–ESI-MS system containing the interface of the monolithic capillary column and the carbon fiber emitter. A relatively narrow scan range in the MS mode (690–691 *m/z*) was employed during data dependent scan MS–MS analysis for the detection of the target peptide. The target peptide, which was spiked in the digested BSA, was successfully detected and identified by tandem MS followed by SEQUEST database search software, on both POS–DVB and PLMA–EDMA columns (Figs. 6 and 7). As low as 500 amol/μl of the target peptide on the POS–DVB column was detected with a signal-to-noise ratio (S/N) of 5 and identified by SEQUEST with a cross-correlation score (X_{corr}) of 3.5, whereas the detection limit on the PLMA–EDMA column was 1 fmol/μl (S/N = 4) and X_{corr} was 4.3. The higher sensitivity from separation on POS–DVB columns was likely a result of its above noted superior separation efficiency. The high sensitivity shown in our system is, at least in part, due to the performance of the carbon fiber emitter [35]. This performance illustrates that an LC–ESI-MS system, containing a monolithic column and a carbon fiber emitter, can be successfully applied to the detection of low-level proteins in the presence of high-abundance proteins in the sample.

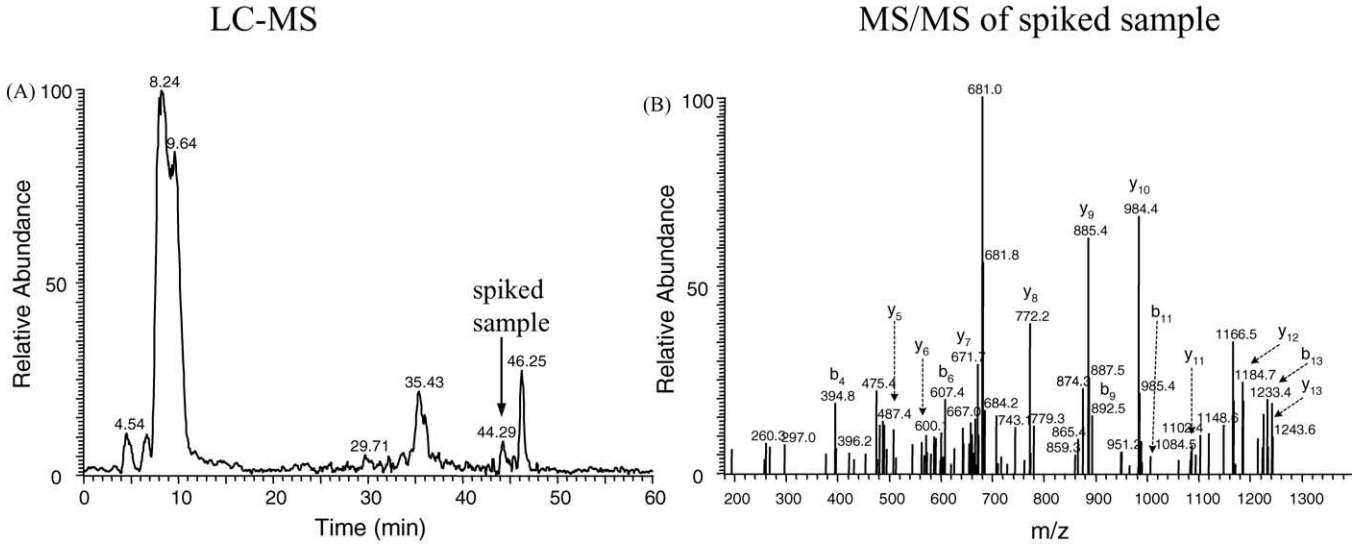


Fig. 7. LC–MS–MS identification of 1 fmol/μl peptide from trypic digested myoglobin which was spiked in 10 μg/μl of digested BSA on a PLMA–EDMA monolithic column (100 μm i.d., 375 μm o.d., 100 mm long). (A) The base peak chromatogram in the scan range of 690–691 *m/z*. (B) The MS–MS spectrum of the detected target peptide (HGTVVLTALGGILK) selected from digested myoglobin. Other conditions are the same as those in Fig. 4.

4. Conclusions

A monolithic capillary column coupled with a carbon fiber emitter for capillary HPLC–ESI-MS has been developed and used for highly efficient separation and sensitive detection of tryptically digested proteins. POS–DVB monolithic column offered better chromatographic performances compared to PLMA–EDMA column, possibly due to superior surface morphology and smaller pore diameter. A carbon fiber emitter interfaced with a capillary monolithic column showed good long-term stability and high sensitivity. Results for the capillary HPLC–ESI-MS of tryptic peptides obtained using the monolithic column coupled with carbon fiber emitter demonstrate the feasibility of separation and highly sensitive detection of proteins for proteomic researches.

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References

[1] P.H. O'Farrell, J. Biol. Chem. 250 (1975) 4007.
[2] A. Görg, C. Obermaier, G. Boguth, A. Harder, B. Scheibe, R. Wildgruber, W. Weiss, Electrophoresis 21 (2000) 1037.
[3] A.J. Link, J. Eng, D.M. Schieltz, E. Carmack, G.J. Mize, D.R. Morris, B.M. Garvik, J.R. Yates III, Nat. Biotechnol. 17 (1999) 676.
[4] D.B. Wall, M.T. Kachman, S. Gong, R. Hinderer, S. Parus, D.E. Misek, S.M. Hanash, D.M. Lubman, Anal. Chem. 72 (2000) 1099.
[5] J. Peng, J.E. Elias, C.C. Thoreen, L.J. Licklider, S.P. Gygi, J. Proteome Res. 2 (2003) 43.
[6] K. Wagner, T. Miliotis, G. Marko-Varga, R. Bischoff, K.K. Unger, Anal. Chem. 74 (2002) 809.
[7] Y. Shen, R.D. Smith, Electrophoresis 23 (2002) 3106.
[8] K.B. Tomer, Chem. Rev. 101 (2001) 297.
[9] F. Svec, J.M.J. Fréchet, Anal. Chem. 64 (1992) 820.
[10] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, Anal. Chem. 68 (1996) 3498.
[11] H. Zou, X. Huang, M. Ye, Q. Luo, J. Chromatogr. A 954 (2002) 5.
[12] C. Legido-Quigley, N.D. Marlin, V. Melin, A. Manz, N.W. Smith, Electrophoresis 24 (2003) 917.
[13] E.F. Hilder, F. Svec, J.M.J. Fréchet, Electrophoresis 23 (2002) 3934.
[14] F. Svec, J.M.J. Fréchet, Chem. Mater. 7 (1995) 707.
[15] C. Viklund, F. Svec, J.M.J. Fréchet, K. Irgum, Chem. Mater. 8 (1996) 744.
[16] S. Zhang, X. Huang, J. Zhang, Cs. Horváth, J. Chromatogr. A 887 (2000) 465.
[17] R. Wu, H. Zou, H. Fu, W. Jin, M. Ye, Electrophoresis 23 (2002) 1239.
[18] R. Shediac, S.M. Ngola, D.J. Throckmorton, D.S. Anex, T.J. Shepodd, A.K. Singh, J. Chromatogr. A 925 (2001) 251.
[19] R. Wu, H. Zou, M. Ye, Z. Lei, J. Ni, Anal. Chem. 73 (2001) 4918.
[20] C. Ericson, S. Hjertén, Anal. Chem. 71 (1999) 1621.
[21] R.E. Moore, L. Licklider, D. Schumann, T.D. Lee, Anal. Chem. 70 (1998) 4879.
[22] S. Zhang, J. Zhang, Cs. Horváth, J. Chromatogr. A 965 (2002) 83.
[23] A. Premstaller, H. Oberacher, W. Walcher, A.M. Timperio, L. Zolla, J. Chervet, N. Cavusoglu, A. van Dorsselaer, C.G. Huber, Anal. Chem. 73 (2001) 2390.
[24] H. Oberacher, C.G. Huber, Trends Anal. Chem. 21 (2002) 166.
[25] W. Walcher, H. Oberacher, S. Troiani, G. Hölzl, P. Oefner, L. Zolla, C.G. Huber, J. Chromatogr. B 782 (2002) 111.
[26] A. Premstaller, H. Oberacher, C.G. Huber, Anal. Chem. 72 (2000) 4386.
[27] P.J. Oefner, C.G. Huber, J. Chromatogr. B 782 (2002) 27.
[28] Y.H. Maa, S. Lin, C. Horváth, U. Yang, D.M. Crothers, J. Chromatogr. 508 (1990) 61.
[29] I. Gusev, X. Huang, C. Horváth, J. Chromatogr. A 855 (1999) 273.
[30] X. Huang, S. Zhang, G.A. Schultz, J. Henion, Anal. Chem. 74 (2002) 2336.
[31] M. Wilm, M. Mann, Anal. Chem. 68 (1996) 1.
[32] J.H. Wahl, D.C. Gale, R.D. Smith, J. Chromatogr. A 659 (1994) 217.
[33] J.F. Kelly, L. Ramaley, P. Thibault, Anal. Chem. 69 (1997) 51.
[34] M. Wetterhall, S. Nilsson, K.E. Markides, J. Bergquist, Anal. Chem. 74 (2002) 239.
[35] J. Liu, K.W. Ro, M. Busman, D.R. Knapp, Anal. Chem. 76 (2004) 3599.
[36] C. Ericson, J.L. Liao, K. Nakazato, S. Hjertén, J. Chromatogr. A 767 (1997) 33.
[37] C.G. Huber, P.J. Oefner, G.K. Bonn, Anal. Biochem. 212 (1993) 351.
[38] C.G. Huber, G. Kleindienst, G.K. Bonn, Chromatographia 44 (1997) 438.
[39] C. Yu, F. Svec, J.M.J. Fréchet, Electrophoresis 21 (2000) 120.
[40] P.J. Dumont, J.S. Fritz, J. Chromatogr. A 691 (1995) 123.
[41] S. Xie, R.W. Allington, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 865 (1999) 169.
[42] B. Mayr, G. Hölzl, K. Eder, M.R. Buchmeiser, C.G. Huber, Anal. Chem. 74 (2002) 6080.