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On-chip solid phase extraction and enzyme digestion using cationic PolyE-323 coatings and porous polymer monoliths coupled to electrospray mass spectrometry

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ABSTRACT

We evaluate the compatibility and performance of polymer monolith solid phase extraction beds that incorporate cationic charge, with a polycationic surface coating, PolyE-323, fabricated within microfluidic glass chips. The PolyE-323 is used to reduce protein and peptide adsorption on capillary walls during electrophoresis, and to create anodal flow for electrokinetically driven nano-electrospray ionization mass spectrometry. A hydrophobic butyl methacrylate-based monolithic porous polymer was copolymerized with an ionizable monomer, [2-(methacryloyloxy)ethyl] trimethylammonium chloride to form a polymer monolith for solid phase extraction that also sustains anodal electroosmotic flow. Exposure of the PolyE-323 coating to the monolith forming mixture affected the performance of the chip by a minor amount; electrokinetic migration times increased by ~5%, and plate numbers were reduced by an average of 5% for proteins and peptides. 1-mm long on-chip monolithic solid phase extraction columns showed reproducible, linear calibration curves (R^2 = 0.9978) between 0.1 and 5 nM BODIPY at fixed preconcentration times, with a capacity of 2.4 pmol or 0.92 mmol/L of monolithic column for cytochrome *c*. Solution phase on-bed trypsin digestion was conducted by capturing model protein samples onto the monolithic polymer bed. Complete digestion of the proteins was recorded for a 30 min stop flow digestion, with high sequence coverage (88% for cytochrome *c* and 56% for BSA) and minimal trypsin autodigestion product. The polycationic coating and the polymer monolith materials proved to be compatible with each other, providing a high quality solid phase extraction bed and a robust coating to reduce protein adsorption and generate anodal flow, which is advantageous for electrospray.

1. Introduction

Microfluidic systems in which multiple sample preparation processes are integrated into the same device may provide a significant advantage in time, speed and automation for proteomics applications [1–3]. Electroosmotic flow (EOF) provides a powerful means of controlling and delivering fluids in an integrated system. We envision a system with electrokinetically delivered protein collection by a solid phase extraction (SPE) column, before or after upstream separation, followed by protein digestion and elution to downstream separation and electrospray mass spectrometry (ES-MS). Such a system requires a number of materials and surface properties be in place within the microfluidic devices, including a cationic surface to generate anodal flow for electrospray when detecting positive ions, a surface that has good properties for electrophoretic separation of proteins and peptides, and an SPE bed that is compatible with the cationic surface in terms of function and fabrication. Here, we evaluate the compatibility and performance of polymer monolith SPE beds that incorporate cationic charge, with a polycationic surface coating, PolyE-323, fabricated within fused silica capillaries and microfluidic glass chips.

Porous hydrophobic polymer monolithic columns for on-chip SPE are receiving increasing attention as an alternative to bead packed beds [4–7], which require retaining frits, and have been reported for both offline and online coupling to ES-MS [4,7–13]. Co-polymerization of ionic functionalities, such as [2-(methacryloyloxy)ethyl] trimethylammonium chloride (META) [14–16] and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) [8,17], with hydrophobic polymerization mixtures have been reported. Proteins adsorbed to commercially available micro-beads in vials or packed in columns [18–21] can be effectively digested while adsorbed, via the addition of trypsin solution. Here we report on META-monolith fabrication on microchip for

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SPE, on-bed trypsin digestion of captured protein, and elution for ES-MS, all driven electrokinetically.

The polymeric amine, PolyE-323, provides a coating that generates sufficient anodal EOF for nano-ES-MS and effectively eliminates protein and peptide adsorption in fused silica capillaries [22,23]. We explore the compatibility of PolyE-323 coatings with META-monolith formation, and the impact of this combination on capillary electrophoresis separation and SPE performance. The integration of electrokinetically driven SPE, on-bed tryptic digestion, elution and ES-MS of several proteins was demonstrated, illustrating that this pairing of organic coatings and beds are suitable for coupling these steps with separation techniques such as CE and CEC.

2. Materials and methods

2.1. Chemicals, materials and microchips

With the exceptions below, all reagents were from Sigma–Aldrich (Milwaukee, WI, USA), and solvents were HPLC or LC-MS grade. Benzoin was from General Intermediates of Canada (Edmonton, AB, Canada). BODIPY 493/503 (4,4-difluro-1,3,5,7,8-penta methyl-4-bora-3a,4-diaza-*s*-indacene) was from Molecular Probes (Eugene, OR, USA). Butyl methacrylate (BMA), ethylene glycol dimethacrylate (EDMA), [2-(methacryloyloxy) ethyl] trimethylammonium chloride (META) and water were purified as described previously [24]. Protein and peptide stock solutions (1–2 mg/mL) were prepared in Milli-Q water and 0.1 mM BODIPY in methanol, stored at −20 °C, then adjusted to room temperature and diluted as required prior to use. All aqueous solutions were prepared with Milli-Q water and filtered through 0.2 μm Nylon syringe filters (Chromatographic Specialties Inc., Brockville, ON, Canada). Fused silica capillary (50 μm i.d., 365 μm o.d.) was from Polymicro Technologies Inc. (Phoenix, AZ, USA).

Corning 0211 glass microfluidic devices were fabricated as previously described [24,25], at the University of Alberta NanoFab (Edmonton, AB, Canada); 3.5 cm long channels were etched 56 μm wide and 20 μm deep, with a 140 μm wide, 1 mm long segment etched at the center of the device, Fig. 1(a), and 1 mm wide access ports on the cover plate. At the end of the channel, a 4 cm long 50 μm i.d. gold coated nano-electrospray, pulled, fused silica capillary tip was inserted, as described previously [25,26]. For CE separation of proteins and peptides as well as measuring the EOF flow velocity of the coated chip using the neutral marker BODIPY, a P/ACE 5010 Beckman Instrument (Fullerton, CA, USA) and a microfluidic device with "double T injector" were used [27], the latter with 10 μm deep and 30 μm wide separation channels.

2.2. Channel coating and photo-polymerized monolith formation

PolyE-323 was synthesized from 1,2-bis(3-aminopropylamino) ethane and epichlorohydrin and channels or capillaries were coated with it according to published procedure [22]. The device was first conditioned with 1 M NaOH for 30 min and rinsed with Milli-Q water for 15 min with suction from house vacuum, then flushed with 7.5% (w/w) PolyE-323 solution (adjusted to pH 7 with acetic acid) for 30 min, then rinsed with 10 mM NH_4Ac (pH 8.0).

The monolith beds were prepared using the polymerization mixture described in Table 1, after sparging with nitrogen for 5 min to remove dissolved oxygen prior to mixing with initiator. The PolyE-323 coated channels were then filled with the polymerization mixture and the access ports were sealed. A transparency mask was used to selectively expose the 140 μm wide, 1 mm long channel segment to the UV source described previously [24].

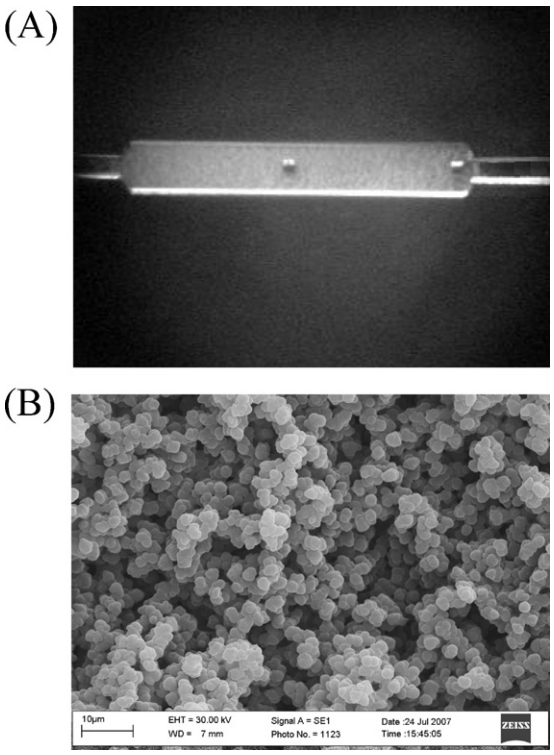

Fig. 1. (A) Optical micrograph of a 1 mm long monolith polymer bed, with 30 μm^2 posts, prepared in 20 μm deep, 140 μm wide microchannel; (B) SEM image of the monolith showing details of nodules and pores. The monolith has sharp edges and uniform structure.

Table 1
Polymerization mixtures and reaction conditions used for the preparation of monoliths.

Polymer	A[a]	B[a]
EDMA, g	0.24	0.24
BMA, g	0.36	0.36
META/H_2O[b], g		0.09
Benzoin, g	0.006	0.006
1-Octanol, g	0.9	
1-Propanol, g		0.54
1,4-butandiol, g		0.27
Exposure time, min	8	4

[a] (A) neutral hydrophobic monolithic polymer and (B) positively-charged monolithic polymer.
[b] META/H_2O: (0.2 g of 75% aqueous META solution/1.8 g H_2O).

Monoliths were then flushed with methanol/water mixture (1:1, v/v) at 0.05 μL/min flow rate for 5 min to remove un-reacted reagents. When not in use, the device was stored in buffer. Scanning electron microscopy (SEM) of the bed cross sections on-chip and mercury porosimetry of bulk monolith materials were done as described previously [24].

2.3. Instrumentation

The computer controlled power supply and relay arrangement has been described elsewhere [28], as has the laser induced fluorescence system [29], which used a 530 nm emission filter. A PE/Sciex API 150 EX single quadrupole mass spectrometer (PerkinElmer/Sciex, Concord, ON, Canada) was operated in positive ion mode. The gold coated nanoelectrospray tip operation was optimized by electrokinetically infusing bradykinin fragment (1-5), positioning it 5–10 mm from the orifice at tip voltages from 3.2 to 3.5 kV. Masses were scanned from *m/z* 500–1200 at 0.5 amu per step with a dwell time of 1 ms, unless otherwise stated.

Peptides were identified by database searching using a combination of ExPASY (http://us.expasy.org/) and MS-Digest of Protein Prospector (http://prospector.ucsf.edu/).

2.4. Chip operation

For CE experiments in the double-T injector chip, sample injection and separation with the push-back method was used, as described previously [28]. Sample injection for 10 s at −0.5 kV preceded separation at −1.0 kV, with −250 V push-back voltages on the sample and sample waste reservoirs. The fluorescence detector was positioned 2 cm from the double-T intersection.

The capacity of monolithic SPE beds was determined using the 50% point (inflection point) on breakthrough curves [30] with 5 μM cytochrome *c*, pumped at an EOF rate of 0.19 μL/min, less the time required for cytochrome *c* to reach the MS in the absence of the monolithic column. For SPE studies, the device was first equilibrated with the loading buffer (10 mM NH_4Ac, pH 8.0, or 5 mM formic acid), then sample, in the loading buffer, was delivered with 2.0 kV between sample and waste reservoirs for a specified period, followed by a 1 min loading buffer rinse. Elution buffer (10 mM NH_4AC/60% ACN or 5 mM formic acid/60% ACN) was then delivered at 2.0 kV.

For trypsin digestion of adsorbed protein, the SPE column was first saturated with the protein of interest (0.124 mg/mL cytochrome *c* or 0.33 mg/mL BSA) in 5 mM formic acid, washed with 20 mM NH_4HCO_3 (pH 8.0) digestion buffer for 5 min with −3.0 kV at the sample reservoir and 3.2–3.5 kV on the electrospray tip. Then 0.31 mg/mL trypsin solution in digestion buffer was delivered with the same voltages and the reaction proceeded for 30 min under stopped flow conditions, then rinsed with 1.1 μL of 5 mM formic acid for 5 min at an EOF rate of 0.22 μL/min. Tryptic peptides were eluted with 5 mM formic acid/60% ACN with the above voltages scheme and detected by ES-MS. Prior to loading BSA, 1 mL of 100 μM BSA was reacted with 250 μL of 45 mM dithiothreitol at 50 °C for 20 min, then capped using 250 μL of 100 mM iodoacetamide at room temperature in the dark for a further 20 min.

3. Results and discussion

3.1. Preparation of porous polymer monolith in microchannel

A 1 mm long SPE bed was prepared within a glass microfluidic chip by UV-initiated polymerization of a methacrylate-based monomer. Polymer monoliths were prepared with two different surface chemistries (Table 1) at the desired location of the microchip. Monoliths prepared with BMA and EDMA are as hydrophobic as C18 beads [16,31], so can be used for SPE of hydrophobic compounds such as proteins. The quaternary ammonium functionality of META supports anodal EOF [14] to drive fluid through the column.

He et al. [24] and Yu et al. [16] have reported several crucial factors for proper performance of monolithic columns in microfluidic devices; sharp column edges to reduce band-broadening effects, high enough porosity to allow the use of reasonably low pressures, and sufficient crosslinking to give bed stability under pressure or over time. He et al. established polymerization compositions using octanol, BMA and EDMA in microchannels with excellent edge definition and high reproducibility [24], and one of those compositions was used here (Table 1). The addition of an ionic co-monomer such as META, however, affects the phase-separation process [32], so that with octanol as a porogen a very dense structure (by SEM) with a high flow resistance to syringe-driven flow was obtained. The binary system of 1-propanol and 1,4-butanediol (Table 1) described by Yu et al. [32] was well suited to the preparation of META-containing monolith, in terms of pore properties and edge resolution, as illustrated in Fig. 1. Mercury intrusion porosimetry of the bulk monolith gave an average pore size of 950 nm and porosity of 62.4%, somewhat lower than the 2–3 μm median pore size reported by Yu et al. [32] for a similar composition. The monolith exhibited sufficient permeability and mechanical stability to allow transport of liquid through it at flow rates of up to 3 μL/min using a syringe pump.

Typically for glass microchips, monoliths are anchored to the channel walls by modifying the channel surface with a silane priming reagent [17,33]. We have instead used changes in the channel geometry to retain the monolith, because the silane priming agent may not be compatible with the PolyE-323 cationic coating. An enlarged bed was designed in the microchannel, and post structures were fabricated at the outlet region of the bed in several design versions. Flows driven by syringe applied pressure or by EOF did not dislodge the monolithic polymers, whether or not posts were added as anchoring structures. Apparently the restriction caused by the narrowing channels at the end of the bed provides sufficient mechanical stability for the monoliths.

3.2. EOF in PolyE-323 coated microchannel

PolyE-323 is an effective coating polymer for capillary electrophoretic separation of proteins and peptides in fused silica capillaries [22]. The polymeric amine coating has a high density of postive charges that chemisorb to the glass surface with its negatively charged silanate groups. The EOF and lifetime of a PolyE-323 coated microchannel was investigated by performing successive EOF measurements using a neutral marker BODIPY over a 20-day period. For 80 runs performed in one day, the EOF was $(4.20 \pm 0.06) \times 10^{-4}$ cm^2 V^{-1} ^{P}s^{-1} (%RSD = 1.4%), similar to that reported for PolyE-323 coated fused silica capillaries [22]. The average values of the EOF measured on the first and last days were $(4.23 \pm 0.03) \times 10^{-4}$ cm^2 V^{-1} ^{P}s^{-1} (n = 10) and $(4.11 \pm 0.06) \times 10^{-4}$ cm^2 V^{-1} s^{-1} (n = 10), respectively, while the average for the 20-day period was $(4.16 \pm 0.08) \times 10^{-4}$ cm^2 V^{-1} s^{-1} (n = 110 runs). Six chips were coated on six different days and their EOF measured 10 times for each chip, giving ±1.8% RSD for the EOF of the six chips, indicating the robustness of the coating procedure.

Long-term electrospray stability of the PolyE-323 coating was studied by electrokinetically infusing bradykinin fragment 1-5 and cytochrome *c* into the MS. The stability of the total ion current (TIC) over a period of 1 h is shown in Fig. 2; satisfactory electrospray stability with RSD's of 4.4% and 8.4% were obtained for bradykinin fragment 1-5 and cytochrome *c*, respectively. The small spikes seen in the TICs are likely due to bubbles, which cause some fluctuation in the ion current [9]. The mass spectra on the right side of Fig. 2 show a single mass peak for bradykinin fragment 1-5 and the protein charge envelope for cytochrome *c*, with no signal corresponding to bleeding of the polymer, indicating good stability of the coating.

3.3. Effect of polymerization mixture on PolyE-323 coating

To investigate the effect of the META monolith polymerization mixture on the performance of the PolyE-323 coating, the mixture was flushed through a coated capillary for 1 min, left in the dark for 8 min, then rinsed away with 50% methanol. Fig. 3 shows the separation of five proteins on a PolyE-323-coated capillary, before and after exposure of the coating to the polymerization mixture. The migration times of the proteins shifted by 3.4%, 3.9%, 4.6%, 5.7% and 5.8% after exposure to the polymerization mixture. Similar shift in migration times (2.8%, 3.6%, 4.0% and 4.4%) were observed for four peptides (methionine enkephalin, angiotensin II, leucine enkephalinamide and somatostatin), data not shown. These slight

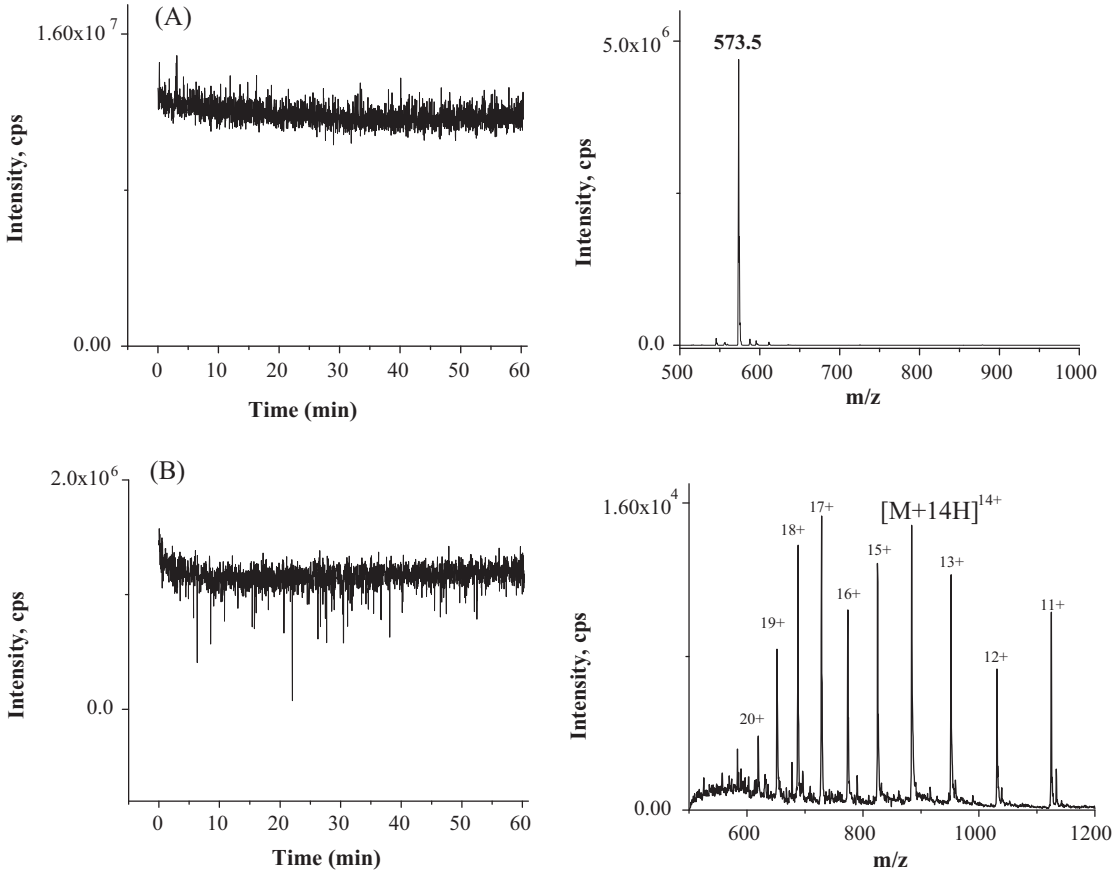

Fig. 2. Total ion chromatogram and mass spectra of 87 μM bradykinin (fragment 1-5) (A) and 20 μM cytochrome *c* (B) in 5 mM formic acid with 25% MeOH. Samples were electrokinetically infused in a PolyE-323 coated microchannel by applying −2 kV at the sample reservoir and 3.2 kV at the electrospray tip.

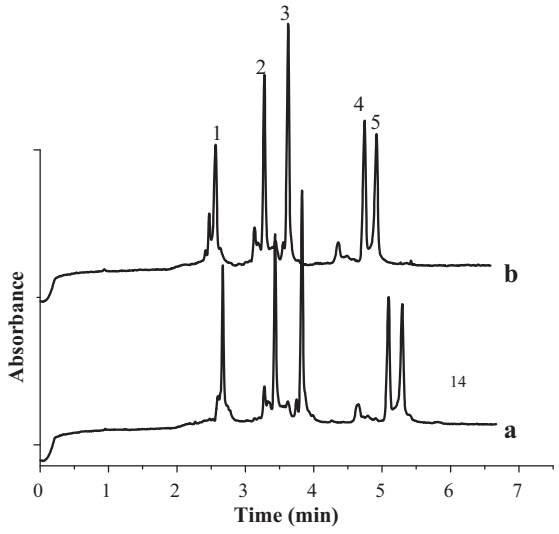

Fig. 3. Electropherograms of a mixture of five proteins obtained using a PolyE-323 coated fused silica capillary before (a) and after (b) the coating came in contact with the polymerization mixture. Peaks are identified as: carbonic anhydrase (1), a-chymotrypsinogen (2), ribonuclease A (3), cytochrome *c* (4) and lysozyme (5). Conditions: Capillary i.d. 50 μm, 365 μm o.d., 27 cm total length and 20 cm to the detection window; sample injected for 2 s by pressure, separation at −10 kV, UV detection at 200 nm, protein concentration 0.25 mg/mL, buffer 50 mM ammonium acetate, pH 5. Traces are offset on the *y*-axis for easy viewing.

increases in migration time may arise from adsorption of positively charged META onto the PolyE-323 coating, which would increase the anodal EOF slightly. The separation efficiency of the Poly E-323 coated channel was 5.0×10^5 plates/m for the basic proteins. The efficiency decreased only slightly (4.0–7.7% for the peptides and 4.1–8.4% for the proteins) after exposure to the monomer mixture. We conclude that PolyE-323 remains a good coating for protein and peptide separation, even after exposure to the monolith-forming mixture.

3.4. Characterization of the monolithic column

Solid phase extraction of neutral BODIPY, which has a high affinity for the monolith, was performed at an EOF rate of 0.19 μL/min for a 30 s preconcentration time, as described in section 2.4, chip operation. Supplementary Figure S1 shows the elution traces of BODIPY and the corresponding calibration curve. A linear response was seen between 0.1 nM and 5 nM with an R^2 of 0.9978, giving a limit of detection (LOD, S/N = 3) of 4 pM obtained by extrapolating the data in Supplementary Figure S1. Using the same detection system, the best LOD we obtained [34] was 30 pM for fluorescein without preconcentration.

The effect of preconcentration time was also evaluated. A 0.5 nM BODIPY solution was concentrated for varying times and a linear relationship ($R^2 = 0.9965$) was observed between the resulting peak areas vs. preconcentration times of 10–270 s (Supplementary Figure S2). The linearity indicated that we were far from the breakthrough volume [35] of the monolithic bed at the concentration of BODIPY used, so that further increasing preconcentration

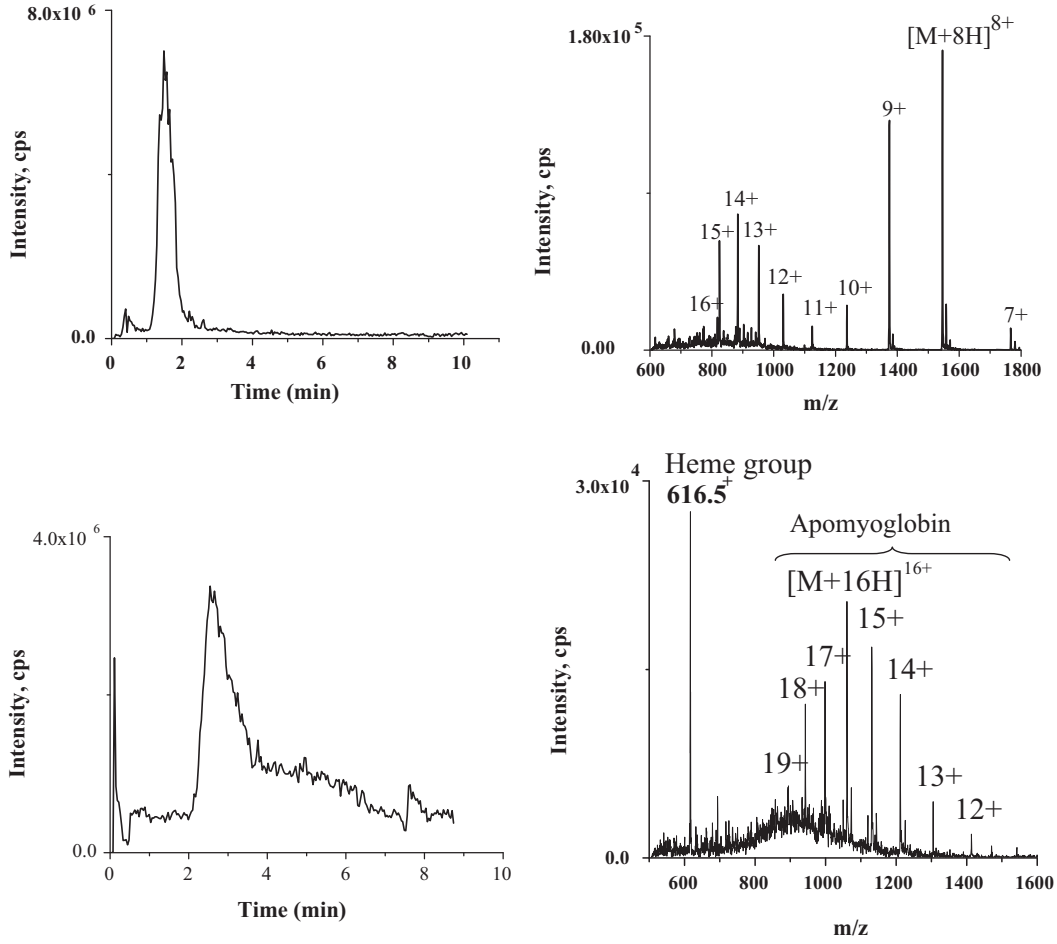

Fig. 4. Total ion electropherograms and corresponding mass spectra of 1 μM cytochrome *c* (upper trace) and 1 μM myoglobin (lower trace) following SPE in 1 mm long monolithic column. Loading buffer: 5 mM formic acid, elution buffer: 5 mM formic acid with 60% ACN. Samples were loaded and eluted electrokinetically with −2.5 kV at the sample reservoir and 3.2 kV on the electrospray tip.

time would enhance signal. A preconcentration factor of 105 was calculated for our monolithic column by loading 20 pM BODIPY for 30 min.

The monolithic bed retains proteins under aqueous conditions, while at 60% ACN, the proteins partition between the hydrophobic bed and the elution buffer, resulting in the elution profiles shown in Fig. 4 for cytochrome *c* and myoglobin. A sharp peak for cytochrome *c* indicates rapid desorption with the elution buffer. The more hydrophobic myoglobin eluted later, with a wider peak and significant tailing, but further optimization of the elution solvent was not pursued. Representative m/z spectra for eluted cytochrome *c* and myoglobin are also shown in Fig. 4. Both are characterized by an envelope of peaks, each peak representing a different charge state of the analyte. For myoglobin, the heme group is non-covalently bound to apomyoglobin, so the peak at 616.5 is due to the heme group, and a set of peaks with multiple charges arise from apomyoglobin. For cytochrome *c*, the heme group is covalently attached, so a free heme group is not observed, and the peaks represent the different cytochrome *c* charge states.

To determine the overall capacity of the monolithic bed for proteins, breakthrough curves were obtained. Values of 30 ng (2.4×10^{-12} mol) of cytochrome *c*, corresponding to a specific capacity of 11.5 mg/mL of bed volume or 0.92 mmol/L of bed were obtained. The extraction capacity of an SPE column depends on a number of factors including the nature of the probe, carbon loading of the sorbent, and the quantity and type of the SPE phase, so comparison to literature values are not exact. Nevertheless, under our conditions, the capacity per unit volume of the META/BMA/EDMA monolithic bed is as good as, or better, than that reported for C18 bead packed beds (0.25 mmol/L for BODIPY) [30] and hydrophobic monolithic columns on-chip [36,37] (e.g. 2.6 mmol/L for imipramine).

3.5. On-chip SPE followed by protein digestion

Protein digestion is a key element in protein identification by MS. Our group [21] as well as others [18–20] have shown that proteins may be concentrated on beads and then digested by solution phase trypsin while still adsorbed, enhancing the kinetics and reducing trypsin autolysis products by effectively increasing the protein concentration. The above reports [18,20,21] used beads packed in microcolumns or microchips, and showed similar performance to the more conventional scheme of digesting solution phase proteins on immobilized trypsin beds. Packed columns, however, require frits to hold the beads. In situ polymerization to form monolithic columns may offer advantages in fabrication, reproducibility and performance compared to packed beds for this application [38].

Fig. 5 shows mass spectra of cytochrome *c* (0.124 mg/mL) and BSA (0.33 mg/mL) digested with trypsin solution after loading onto a monolithic column from an aqueous solution at an EOF rate of 0.22 μL/min. To reduce the detection of trypsin autolysis byproducts, the monolithic column was rinsed with 5 mM formic acid prior to elution of the peptide digests. Three cytochrome *c* peptide peaks at *m*/*z* of 562.5, 589.5 and 817.5 were observed during

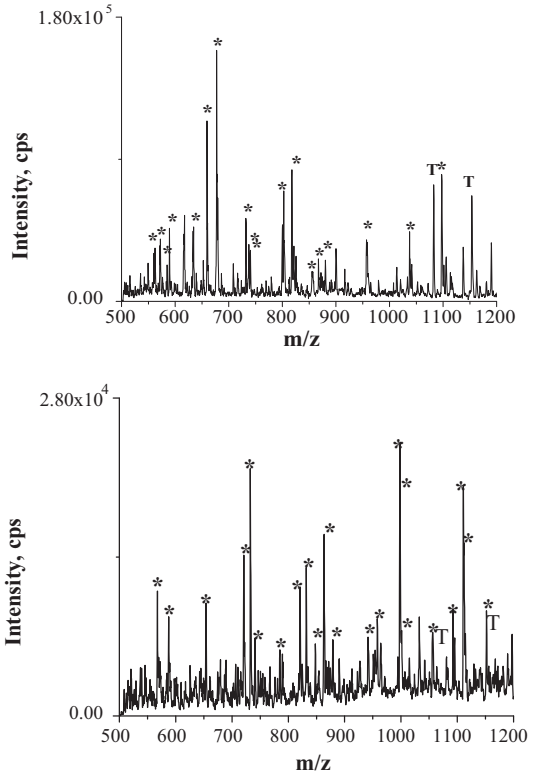

Fig. 5. On-chip SPE of 0.124 mg/mL cytochrome *c* (upper trace) and 0.33 mg/mL BSA (lower trace) on a 1 mm long monolithic bed, followed by addition of 0.31 mg/mL trypsin solution to the bound proteins. "*" indicates peaks that can be assigned to digested proteins and "T" indicates peaks due to trypsin autodigestion. See Experimental section for further details.

the washing step. Nonetheless, it is apparent from Fig. 5 that high quality mass spectra were obtained during the subsequent organic elution step, from which the proteins can easily be identified, with little or no trypsin autolysis and no unreacted protein signals detected. During the elution step, eighteen peptide fragments with sequence coverage of 88% were obtained for cytochrome *c* and 22 peaks were identified for the much larger BSA, with sequence coverage of 56%. These results are comparable to reported sequence coverage for cytochrome *c* (82.7–91%) and BSA (69%), obtained in bead-based microcolumns [20,21]. Wang et al. [21] noted that there were as many trypsin autolysis products as there were for digested cytochrome *c* when the protein was adsorbed onto bead based SPE bed and digested with solution phase trypsin. Here we observed only two peaks associated with trypsin autolysis product, as a result of the stop-flow digestion and washing scheme followed.

4. Conclusions

The combination of an *in situ* preparation of a mixed hydrophobic-cationic monolith and polycationic coating of microfluidic channels allows the integration of an electrokinetically driven on-chip SPE preconcentration of proteins, on-chip tryptic digestion and on-line ES-MS detection of the resulting peptides, offering a potential for creating a highly efficient protein sample processing platform for automated MS analysis in proteomic studies. The use of photolithographic patterned monolith, together with voltage driven fluid manipulation, will allow highly multiplexed microfluidic systems for high-throughput sample preparation.

Conflict of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.04.027.

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