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Protein proteolysis and the multi-dimensional electrochromatographic separation of histidine-containing peptide fragments on a chip

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Abstract

This paper reports a system for three-dimensional electrochromatography in a chip format. The steps involved included trypsin digestion, copper(II)-immobilized metal affinity chromatography [Cu(II)-IMAC] selection of histidine-containing peptides, and reversed-phase capillary electrochromatography of the selected peptides. Trypsin digestion and affinity chromatography were achieved in particle-based columns with a microfabricated frit whereas reversed-phase separations were executed on a column of collocated monolithic support structures. Column frits were designed to maintain constant cross sectional area and path length in all channels and to retain particles down to a size of 3 μm. Cu(II)-IMAC selection of histidine-containing peptides from standard peptide mixtures and protein digests followed by reversed-phase chromatography of the selected peptides was demonstrated in the electrochromatography mode. The possibility to run a comprehensive proteomic analysis by combining trypsin digestion, affinity selection, and a reversed-phase separation on chips was shown using fluorescein isothiocyanate-labeled bovine serum albumin as an example.

Keywords: Chip technology; Electrochromatography; Proteomics; Collocated monolithic support structures; Immobilized metal affinity chromatography; Monolithic columns; Proteins; Peptides

1. Introduction

The development of microfluidic analytical systems for the analysis of biological samples is the current focus of many investigators, particularly in relation to the separation of proteins [1], peptides [2], and polynucleotides [3]. Advances in sample injection technology [4], channel fabrication techniques [2], photolithographic synthesis of monolithic columns [5], and the preparation of new stationary phases [6] are driving efforts to build integrated analytical systems that execute the total analysis of samples. These ''micro-total analysis systems'' (μTAS) will perhaps play a major role in the future of life science oriented research and development.

Integrating the multiple unit operations in an analytical process is often difficult, even in a conventional system as seen in the case of proteomics. Samples from biological extracts can be so complex that multiple dimensions of separation are necessary before individual components can be identified and quantified. Moreover, protein identification on a

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global scale is frequently achieved through peptide cleavage fragments. The requisite proteolysis of protein parents is generally preceded by reduction and alkylation of their disulfide bridges to enhance cleavage. Many times the entire sample is digested first, before any type of separation is undertaken. A variety of approaches to these problems have now been described [7].

However, the integration problem in proteomics goes beyond the question of how to physically couple the unit operations, or analytical dimensions, as they will be referred to here. There are much more serious issues of solvent and reagent compatibility between the analytical dimensions, how to select and transport analytes and solvents, and how to minimize analyte dilution and band spreading in the case of coupled separation systems. These problems have generally been addressed in conventional instrumentation with switching valves, concentrator columns, and multiple mechanical pumping systems that drive large volumes of reagent and washing solvents through the system. Integration of at least five analytical dimensions has been achieved in the analysis of human blood serum using a conventional multi-dimensional liquid chromatograph coupled to a mass spectrometer [8]. Unfortunately the capability afforded by having multiple ten-port switching valves, four or more pumps, and the possibility of selecting between six and eight solvents in conventional multi-dimensional liquid chromatography equipment is not yet available in miniaturized systems. At issue today is how these operations will be achieved in a miniaturized format.

Sample complexity is another issue in proteomics. A number of recent papers have described the advantages of using some type of affinity selector to simplify mixtures and concomitantly enhance peptide identification [9,10]. One approach is to use an antibody to select a specific family of proteins from blood samples [11]. After tryptic digestion of the proteins captured by the immunosorbent, peptide fragments can then be generated and sequenced to identify the selected proteins. Avidin-based affinity capture of cysteine-containing peptides that have been alkylated with a reagent containing biotin is a similar, but broader strategy [12,13]. Use of copper loaded immobilized metal affinity chromatography (IMAC) columns to capture and identify histidine-containing peptides is still another paradigm for group specific selection [14]. Finally, lectins have been used to capture and identify glycopeptides based on glycan structure [15]. These affinity selection methods have many advantages. Among the more important are that they allow specific selection of a class of analytes while substantially reducing sample complexity, concentrating the targeted ligands, and permitting peptide sequence to be acquired on the captured species.

Two types of IMAC have been applied to proteomics. One is the enrichment of phosphorylated peptides with Ga(III) or Fe(III) loaded IMAC columns [16]. The other is the selection of histidine-containing peptides with Cu(II) loaded columns [14]. A recent report has shown that 40 μm particles of a chromatographic packing material with a metal chelating stationary phase can be trapped in a microfabricated weir with a 10 μm outlet and after loading the IMAC sorbent with Ga(III) can be used to select phosphopeptides from a 2.4 μl sample introduction port [17]. Peptides released from the sorbent by a pH change were then separated by capillary electrophoresis.

The objective of the study described here was to explore the possibility that trypsin digestion of proteins, affinity selection, and reversed-phase chromatography could be achieved in an integrated, electroosmotically driven, microfabricated system. Microfabricated frits were used to retain immobilized trypsin and a Cu(II) loaded IMAC sorbent. This allowed protein digestion and the capture of histidine-containing peptides on the same chip. Further fractionation of the selected histidine-containing peptides was achieved on a collocated monolithic support structure (COMOSS) column microfabricated in poly(dimethylsiloxane) (PDMS).

2. Experimental

2.1. Reagents

Sylgard 184 PDMS base polymer and curing agent were purchased from Dow Corning (Midland, MI, USA). Hydrochloric acid was bought from Fisher (Fair Lawn, NJ, USA). *N*-acetylcysteine, 2-acrylamido-2-methylpropanesulfonic acid (AMPS),

(3-aminopropyl)triethoxysilane (APTS), copper(II) sulfate pentahydrate, dithiothreitol, ethylenediamine tetraacetic acid (EDTA), fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA), glutaraldehyde, γ-glycidoxypropyltrimethoxysilane (GPS), iminodiacetic acid (IDA), iodoacetic acid, sodium cyanoborohydride, Tris hydroxide, Tris hydrochloride, trypsin, and urea were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium hydroxide was from J.T. Baker (Phillibsburg, NJ, USA). Sodium acetate, sodium chloride, potassium phosphate dibasic and potassium phosphate monobasic were purchased from Malinckrodt (Paris, KY, USA). Ammonium cerium(IV) nitrate was obtained from Lancaster (Pelham, NH, USA). The peptide H–Leu–Gly–His–Gly–Pro–OH was from BACHEM Bioscience (King of Prussia, PA, USA). Fluorescein-5-isothiocyante (FITC isomer I) was purchased from Molecular Probes (Eugene, OR, USA). FITC-BSA digest and FITC labeling of peptides was performed as previously described [18].

2.2. Preparation of PDMS chips

PDMS replicas were molded from a master, or negative relief of the microfabricated system desired as previously described [18,6]. The chip was designed with a COMOSS separation column (4.0 cm long, 5×5 μm COMOSS particles, and 3 μm wide channels), as well as frits for containing sorbents and multiple injection crosses (Fig. 1). After oxidation, the molded PDMS column and cover slab were brought into contact to make an irreversible seal. The channels were modified with AMPS, with an ammonium cerium(IV) nitrate catalyst as described in the literature [6].

2.3. Modification of silica sorbents

Silica (5 μm) was modified with IDA following the procedures of Bogart [19] and Anspach [20]. Silica (5.0 g) was added to a mixture of 5.0 ml of a GPS and 100.0 ml of acetate buffer solution (10 m*M*, pH 5.5). Following degassing, the mixture was heated to 90 °C and kept on a shaker for 6 h. The modified silica was retrieved by suction filtration and rinsed with 300 ml of acetate buffer. IDA (3.33 g) was added to a mixture of 1.0 g of NaOH in 15 ml of water. After adjustment to pH 8.3 with 2.5 *M* NaOH, the GPS modified silica was added and the mixture was degassed, then shaken for 24 h while heated at 60 °C. The product was isolated with suction filtration and rinsed three times with 150 ml of water. The sorbent was suspended in 0.01 *M* HCl at 50 °C for 3 h to convert remaining epoxy groups to diols. The IDA modified particles were retrieved by suction

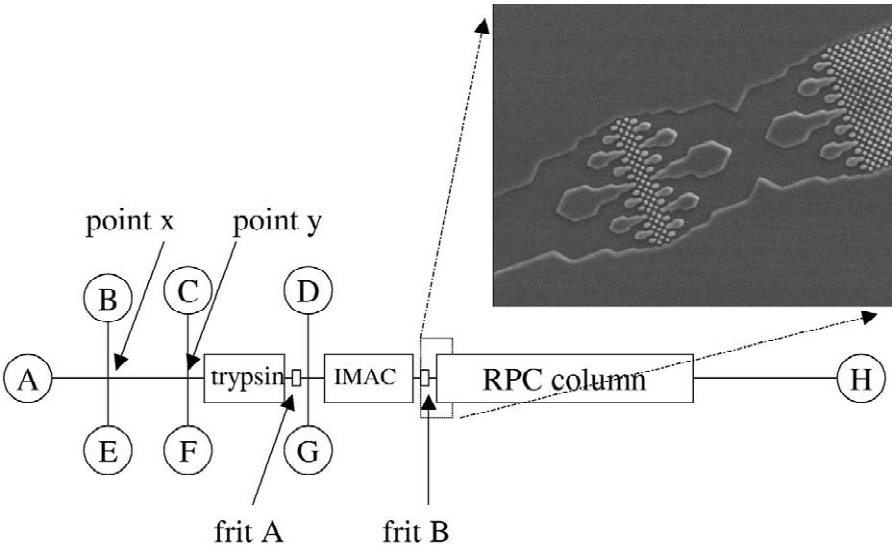

Fig. 1. Scheme of the column used and SEM of the microfabricated frit (frit B) and head of the COMOSS column.

filtration and rinsed with 150 ml water three times. Copper loading was achieved by the incubation of IDA particles in 1 *M* $CuSO_4$ for 12 h.

Silica (5 μm) was modified with trypsin following the procedures of Singh et al. [21] and Applied Biosystems [22]. First 0.5 g of silica were incubated in 100 ml of solution of 10% (v/v) APTS in water that was adjusted to pH 7 with glacial acetic acid, at 80 °C for 3 h. Following suction filtration and rinsing with water the particles were soaked in a 10% (v/v) glutaraldehyde, water solution at room temperature for 1 h. Particles were once again retrieved by suction filtration and rinsed with 150 ml of water three times. Then, 10 mg of trypsin was dissolved in 5 ml of water and added to 2.5 ml of a 1.5 *M* Na_2SO_4, 100 m*M* phosphate buffer (pH 7.4). Following the addition of $NaCNBH_3$ to a final concentration of 5 mg/ml to the solution, the glutaraldehyde modified silica particles were added and mixed on a rocker for 1 min. Then, 1 ml of 1.5 *M* Na_2SO_4, 100 m*M* phosphate buffer (pH 7.4) was added every 5 min to reach a final salt concentration of 1.1 *M*. The mixture was rocked for 90 min at room temperature. Following suction filtration the particles were resuspended in 10 ml 0.2 *M* Tris pH 7.2 with 5 mg/ml $NaCNBH_3$ and rocked for 30 min at room temperature. The media was filtered and washed sequentially with 50 ml of 10 m*M* phosphate buffer pH 7.4 with 150 m*M* NaCl, 50 ml of 1 *M* NaCl, and again with the 50 ml of 10 m*M* phosphate phosphate pH 7.4 with 150 m*M* NaCl.

2.4. Loading and running chip

To load Cu(II)-IMAC particles in frit B (Fig. 1), a slurry of the sorbent in 1 m*M* phosphate buffer pH 7.0 was loaded into well G. This was followed by the application of 5000 V to an electrode in well G and grounding the electrode placed in well H. Trypsin particles were loaded into frit A in the same fashion with the exception that the slurry and the electrode with 5000 V were placed in well F.

Cu(II)-IMAC particles were equilibrated with loading buffer (0.02 *M* phosphate with 0.5 *M* NaCl, pH 7.2). Samples, in loading buffer, were placed in well B and loaded onto the Cu(II)-IMAC particles by applying 2000 V on an electrode in well B and placing a ground electrode in well H. Well B was rinsed with 1 m*M* phosphate buffer three times to clean sample out of well. Buffer was run through all channels for 15 min to wash all unbound sample from the chip.

Peptides were eluted from the Cu(II)-IMAC column with a plug of eluting buffer (0.02 *M* phosphate with 0.5 *M* NaCl and 1 *M* EDTA, pH 9). The eluting buffer plug was formed by an electrokinetic gated flow injection [23], i.e. placing 300 V on wells A and E, and grounding wells B and C. Well E was switched to ground for 0.25 s and then returned to a potential of 300 V for 1 s. After this, all electrodes were switched to ground and removed. A potential of 2000 V was applied to well E and a ground electrode was placed in well H to elute the bound peptides and separate them in the COMOSS column.

Off-chip Cu(II)-IMAC was performed by the same procedure as on chip with the exceptions of packing the 5 μm IDA particles in a 30 mm×2.1 mm I.D., and the manipulation of fluidic movement was via pressure. Off-chip reversed-phase separations were run on a C_{18} silica column (250 mm× 1 mm I.D.) from Alltech (Deerfield, IL, USA). Then, 5 n*M* of sample was injected onto the column and eluted with an 80 min gradient from 100% buffer A (water with 0.01% acetic acid) to 100% buffer B [95% acetonitrile (ACN), 5% water, 0.01% acetic acid].

2.5. Instrumentation

Visualization of fluid movement in channels was achieved with a Nikon Inverted Eclipse TE-300 optical microscope via a TE-FM confocal-fluorescence system and fluorescent markers (1×10^{-4} *M*) as previously described [24]. Data collection was obtained with an epi-fluorescence system built in the laboratory as previously described [25]. HPLC separations were performed on an Integral Microanalytical Workstation from Applied Biosystems (Framingham, MA, USA). Mass spectrometry was done on a QSTAR Workstation from Applied Biosystems.

3. Results and discussion

3.1. Chip architecture

Chip design was driven by the need for multitasking and multi-dimensional separations with

multiple solvents to achieve both proteolysis and peptide analysis. Moreover, there was the need to move a variety of solutions between multiple reservoirs on the chips. It was decided that electroosmotic pumping provided the simplest solvent transport system. This decision directed many of the other design features. The negative aspect of this decision was the risk of overheating when pumping the high ionic strength mobile phase used to elute the IMAC column. A mechanism had to be developed to deal with this problem. Another issue was how to prepare three different columns in a single analytical device. It was concluded that it would be less time consuming to use pre-prepared particulate supports in the trypsin and IMAC columns because the requisite modification procedures to produce bulk sorbents are well known. To this end, it was apparent that frits must be fabricated in the channels on a chip to trap these sorbents and that separate access channels for the introduction of particulate media, sample loading, and elution would be required. Finally, there was the question of how to apply a spatially specific reversed-phase coating in the microfabricated column [26].

The system chosen to solve these problems is outlined in Fig. 1. Procedures for loading columns with packing materials, the coating protocol, and operation are described in the Experimental and Results and discussion sections of the paper.

3.2. Frit design

One of the problems with preparing packed columns on chips is the difficulty of creating a frit to localize the media inside a tubular capillary [27,28]. It has been demonstrated that microfabricated collocated structures can serve as supports for reversed-phase liquid chromatography columns [2], beds for static mixers [24], and filters for trapping contaminants [29]. The fact that microfabricated filters can trap particulate contaminants ranging down to 2 μm in size was exploited in this work to trap chromatography packing materials. One of the desirable features of COMOSS as particle traps is that there is flow around all the particles in the bed. This minimizes stagnant mobile phase mass transfer problems in the particle bed that are experienced in particles trapped behind a weir [17]. Distribution of the flow around the particle in a bed also minimizes bubble formation in electroosmotically driven flow. Still another advantage of a COMOSS trap is that channel width through the trap can be varied independent of the dimensions of the monolithic support structures with no loss of efficiency. Since channels of roughly 1 μm have been fabricated between COMOSSs, it would be possible to trap particles down to 1.5 μm in columns if needed.

A scanning electron microscopy (SEM) image of a 10 μm deep COMOSS frit with monolith dimensions of 5×5 μm and 3 μm wide channels is shown in Fig. 1. A frit of these dimensions will trap all particles with a diameter larger than 3 μm. Because particles often contain microparticulates it is unavoidable that some of them will pass through the COMOSS frit. However, this poses no problem when the rest of the channels in the system are equal to or larger than channels in the frit. Particles that have passed through the frit will be carried through the separation column to the waste well.

3.3. On-chip Cu(II)-IMAC

Cu(II)-IMAC particles of 5 μm in diameter were loaded into the IMAC column by applying a potential in well G and placing a ground electrode in well H (Fig. 1). Trapped particles are seen in Fig. 2a. Analyte transport into the bed was achieved by a combination of electrophoresis and electroosmosis. Fluorescently labeled pentapeptide FITC-LGHRP was electrophoresed into the particle bed where the histidine residue bound to the chelated Cu(II) as seen by the increase in fluorescence intensity on the particles over time (Fig. 2b). Washing the column with buffer did not diminish the level of fluorescence (Fig. 2c), meaning that the histidine-containing peptide had formed a stable complex with the IMAC sorbent. Passage of a pulse of eluting buffer through the Cu(II)-IMAC particles (Fig. 2d) partially stripped the histidine-containing peptide from the column as seen with the line profile of the fluorescence intensity across the particles (Fig. 2e). More than half of the peptide was eluted from the IMAC particles by injection of a pulse of eluting buffer.

It should be noted that the eluting buffer is not injected in a plug as one would typically see in the gated injection mode. Because of the relatively high ionic strength of the eluting buffer, continued application of voltage across the IMAC column filled with

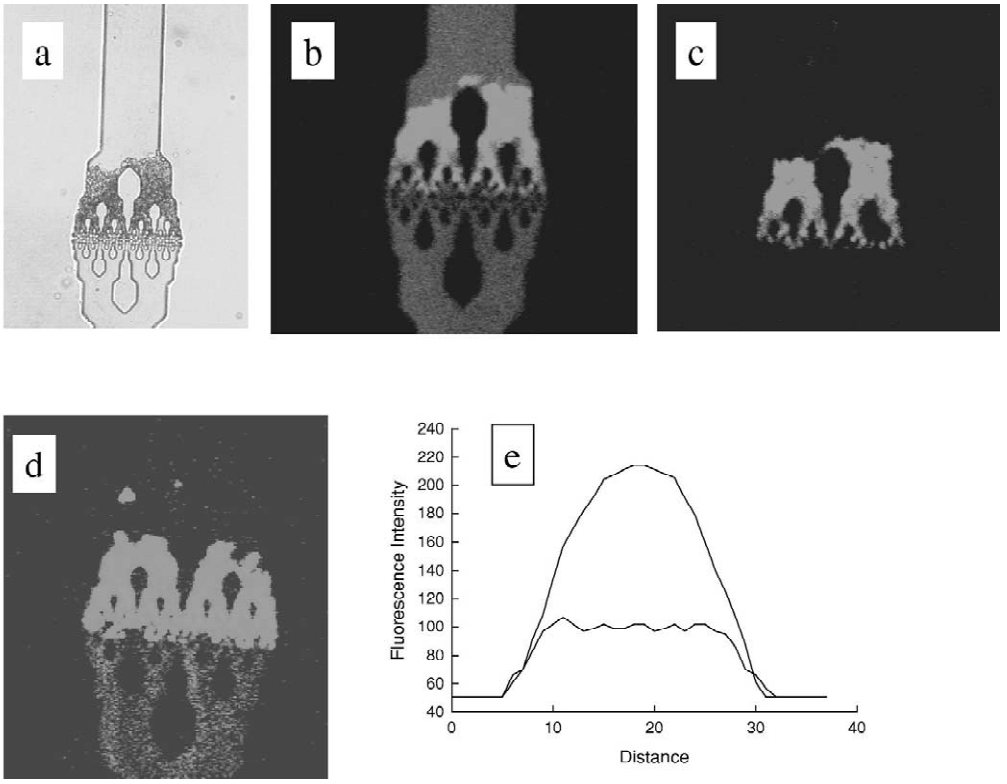

Fig. 2. Optical microscopy images taken with a charge-coupled device (CCD) camera of a microfabricated frit with trapped Cu(II)-IMAC modified particles (a), during the sample loading stage (FITC–Leu–Gly–His–Gly–Pro–OH) (b), washing stage (c), eluting stage (d), and a line profile of fluorescence intensity (e) across the loaded particle layer after loading (top curve) and after injection of an eluting plug (bottom curve).

this solution led to bubble formation that terminated the run. Also, back pressure created from the packed particles caused difficulty in establishing consistent flow. Consequently, eluting buffer was injected as a pulse instead of the more common continuous mode. In essence, this approach treats the top of the separation system containing wells A, B, C, and E, and the channels that connect them as a single cross network by itself. If a gated flow is formed by applying a potential of 300 V on wells A and E, and grounding wells B and C, an injection can occur into the channel between points *x* and *y* (Fig. 1) by switching well E to ground for 0.25 s and then returning the potential to 300 V. When the potential is removed before the plug of eluting buffer passes point *y* it will be confined to the channel between points *x* and *y*. In this way, a plug of eluting buffer was injected without interference from the packed particles. In addition, this step can be done at low potential (200 V/cm) within 30 s, to avoid bubble formation.

Once the eluting buffer plug was injected into the channel between points *x* and *y*, potential on wells A, B, and C was removed and well H was grounded. A potential of 2000 V (500 V/cm) was then placed on electrode E. This caused the eluting buffer plug to migrate through the Cu(II)-IMAC bed and release the bound peptides. Higher voltage was used in this case because none of the channels filled with the high ionic strength eluting buffer experience the applied potential.

Fig. 3 shows the chromatogram of FITC-LGHGP after elution from the IMAC particles through the AMPS-modified COMOSS reversed-phase column

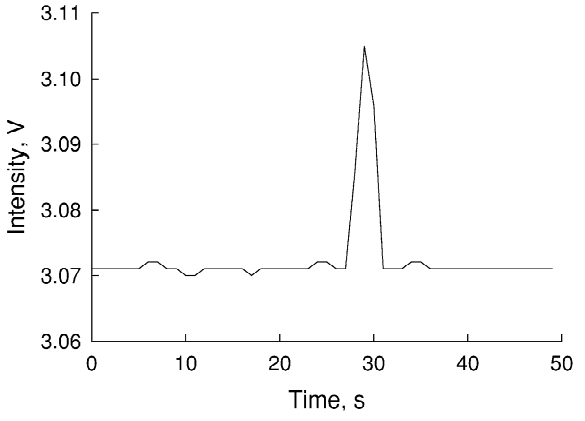

Fig. 3. Resulting chromatogram of FITC–Leu–Gly–His–Gly–Pro–OH following elution from IMAC particles through an AMPS modified COMOSS column. Separation conditions: 1 m*M* phosphate buffer (pH 7.0), 500 V/cm.

(500 V/cm). Only one peak appeared in the chromatogram. This peak is the single histidine-containing peptide present in the loaded sample. When no Cu(II) was loaded on the column, no peaks were observed. This confirms that Cu(II) was essential for capture.

FITC-BSA was chosen as a model system for further evaluation of immobilized metal affinity electrochromatography. Typically, BSA yields 17 histidine-containing peptides when there are no missed cleavages. However, the BSA used in this experiment has an average of 12 FITC labels randomly attached to lysine residues in the protein. Trypsin will not cleave BSA at residues modified with FITC. This reduces the number of peptides in the digest. The laser-induced fluorescence detection system used in these studies detected only histidine-containing peptides with FITC-modified lysine. Because it is not known which lysine residues are modified, it has not been possible to predict the number and identity of peptides appearing in the resulting chromatogram. A separation of tryptically digested FITC-BSA without affinity selection is shown in Fig. 4a. In this electrochromatogram, 11 peaks can be seen. After Cu(II)-IMAC selection, the complexity of the sample mixture is reduced (Fig. 4b).

The same separation was examined with a conventional HPLC system. A C_{18} reversed-phase column (250×1.0 mm I.D.) was used for the separation. Similar to the capillary electrochromatographic (CEC) separation on the chip (Fig. 4a), the reversed-phase HPLC separation of the FITC-BSA digest produced 11 major peaks (Fig. 5a). After histidine selection with Cu(II)-IMAC the number of observed peaks is significantly reduced (Fig. 5b). Histidine-containing peptides were eluted from the Cu(II)-IMAC column (30 mm×2.1 mm I.D.) with the same mobile phase that was used in the miniaturized system. It was possible to identify only 4 FITC-containing peptides that belong to BSA by mass spectrometry. Other peaks in Fig. 5b were unidentifiable, probably due to chymotryptic cleavage and admixture in BSA. However, all peptides found contained histidine.

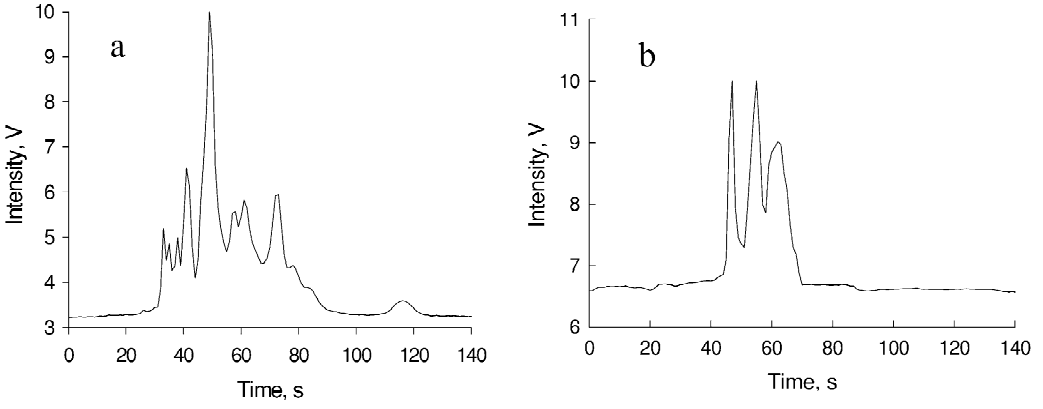

Fig. 4. On chip separation of FITC-BSA digest before (a) and after (b) Cu(II)-IMAC selection. Conditions as in Fig. 3.

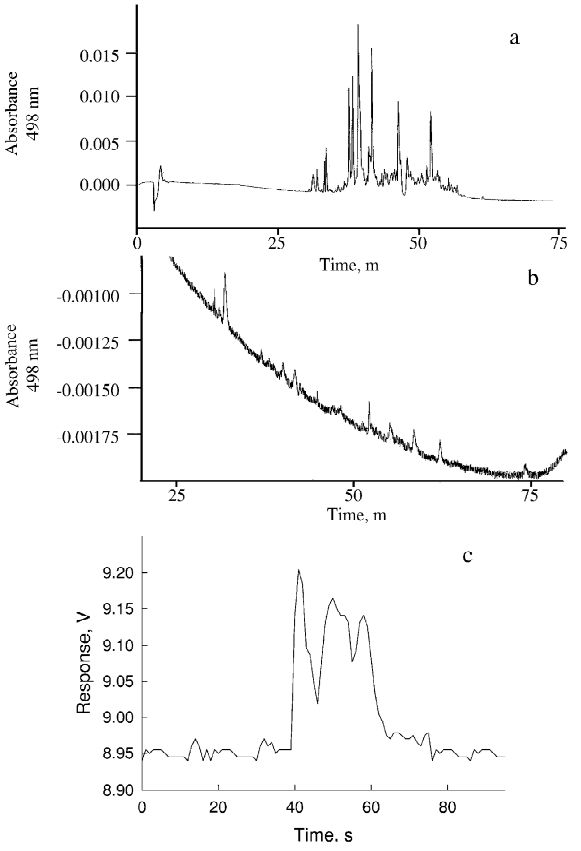

Fig. 5. Off chip separation of FITC-BSA digest before (a) and after (b) Cu(II)-IMAC selection; conditions as in Section 2.4. (c) On chip separation after off chip selection; conditions as in Fig. 3.

For further validation, a sample of histidine-containing peptides eluted from a conventional Cu(II)-IMAC column was injected on an AMPS-modified COMOSS column and an electrochromatogram similar to the one obtained with on-chip affinity selection (Fig. 4b) was observed (Fig. 5c). Resolution in the last case is higher because of a lower concentration of peptides in the sample mixture. The slightly decreased resolution in the on-chip two-dimensional experiment is probably caused by a relatively longer injected plug length. It is evident from shoulders on the first and second peaks that there are more peptides present than observed in the on-chip selection. This is consistent with the HPLC separation and suggests that the affinity selection procedure for histidine-containing peptides on the chip is comparable to conventional HPLC.

3.4. On-chip trypsin digestion

It has been previously shown that on-chip trypsin digestion in both solution (sample well) and with an immobilized enzyme is possible [30,31]. But column digestion has several advantages. One is less auto digestion of trypsin. A second is that there is less sample handling and contamination when coupling to other devices. Finally, there is the higher relative concentration of enzyme in the immobilized enzyme mode. It was the goal of the studies described below to couple on-column proteolysis with a multi-dimensional separation in a μTAS format.

A continuous flow mode of on-column digestion was chosen instead of stop flow for two reasons. First, stopping the flow while the proteins are in the immobilized trypsin bed does not increase the degree of proteolysis [32]. Second, there is no need to synchronize the interruption of potential with the time samples are inside the trypsin bed.

The injection of reduced and alkylated FITC-BSA onto a reversed-phase COMOSS column produces one major peak (Fig. 6a). In contrast, multiple peaks are observed after the same protein was passed through the immobilized trypsin bed and then separated (Fig. 6b). It is evident from the increase in complexity of in the second chromatogram that digestion took place during passage through the immobilized enzyme column.

The on-chip BSA digestion in this study appears to be less complete then in a standard packed column. This is thought to be due to derivatization of BSA with FITC in this case. As was pointed out before, FITC adds to lysine residues and partially blocks trypsin proteolysis.

3.5. Integrating proteolysis, affinity selection, and reversed-phase chromatography

The chip used in this work was designed with multiple frits fabricated in series, that makes it possible to have multiple columns and perform more than one sample manipulation step. Trypsin particles were retained in the column above frit A whereas Cu(II)-IMAC particles were retained above frit B (Fig. 7a). When executing an analysis, reduced and alkylated FITC-BSA was transported through the trypsin digestion bed and eluted directly into the

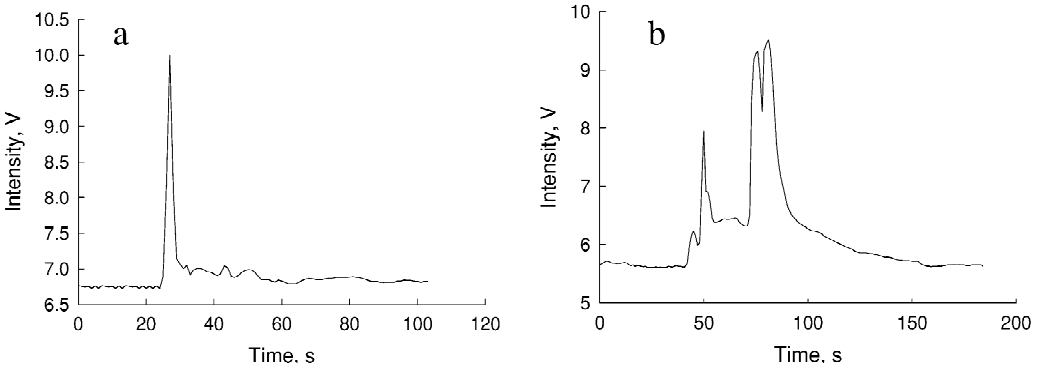

Fig. 6. Chromatogram of FITC-BSA without (a) and after (b) on chip digestion. Separation conditions as in Fig. 3.

Cu(II)-IMAC bed. Upon elution with EDTA, histidine-containing peptides are striped from the IMAC particles and separated in the AMPS-modified RP-CEC column. This is the first example of three-dimensional chromatography on a chip that combines trypsin digestion, affinity selection, and a reversed-phase separation. It is also important to point out that all steps including particle loading, sample digestion, and the separation steps were achieved with electro-osmotic pumping.

The resulting chromatogram shown in Fig. 7b is not the same as that seen in the previous off-chip digestion and on-chip affinity selection, probably due to incomplete proteolysis. Partial proteolysis produces larger peptides that are retained longer, such as with the third peak at 110 s. There is no peak in the chromatogram at 25–35 s, thus, no undigested FITC-BSA remained after passage through the immobilized trypsin layer. Another reason for increasing retention times is due to the lower rate of flow in the longer, multicolumn arrangement.

Unfortunately it was found troublesome to couple PDMS separation systems with electrospray ionization mass spectrometry. Due to severe bleeding of polymer from PDMS, it is much more difficult to couple a separation to the mass spectrometer than simple direct infusion of sample [33,34]. As a conclusion, due to the cost effectiveness and ease of PDMS chip fabrication, it was used for development of the separation system that can be considered as

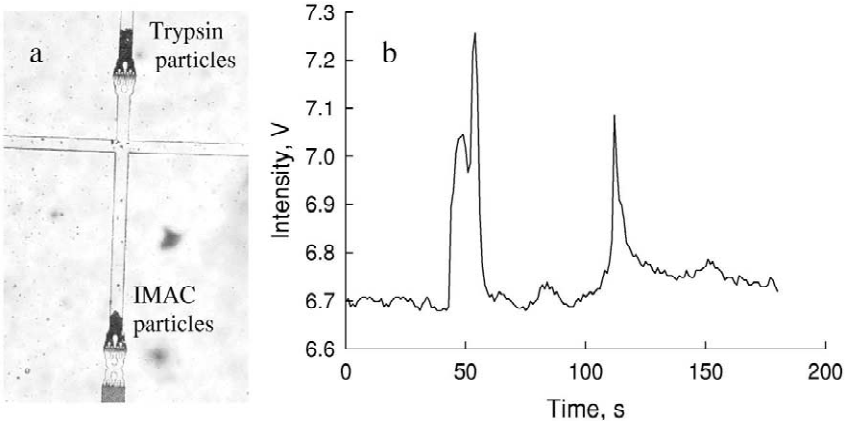

Fig. 7. Optical microscopy image taken with a CCD camera of microfabricated frit A with trapped immobilized trypsin particles and frit B with trapped Cu(II)-IMAC particles (a). A reversed-phase CEC separation of FITC-BSA after on chip trypsin digestion and Cu(II)-IMAC selection (b); conditions as in Fig. 3.

preliminary experiments, or when laser induced fluorescence detection is acceptable. However, the microchip substrate should be changed to quartz or glass for coupling to ESI-MS for real proteomic analysis.

4. Conclusions

It may be concluded from data presented in this paper that at least three-dimensions of sample treatment and separation are possible in an electroosmotically driven, microfabricated device. Fluidic manipulations including loading media, sample injection, and sample elution can be successfully performed by voltage manipulation alone. When applied to protein analysis, this multi-dimensional device was able to achieve the steps of proteolysis, affinity selection of histidine-containing peptides, and reversed-phase chromatography. Moreover, it can be concluded that an important reason for the success of this device was the microfabricated frits that retained microparticulate sorbents. Five μm particle diameter sorbents were retained with ease, whereas retention of particles ranging down to 2–3 μm in diameter will be possible through the fabrication of smaller channels. It is further concluded that the problem of heat production with high ionic strength eluents in CEC can be circumvented in the case of IMAC through the injection of pulses of desorbing agents. There is no need to completely fill channels with high conductivity reagents. Through this protocol it was possible to perform Cu(II)-IMAC on a chip for the selection of histidine-containing peptides with subsequent direct transfer to a reversed-phase chromatography column.

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References

[1] D. Duffy, J.C. McDonald, O.J.A. Schueller, G.M. Whitesides, Anal. Chem. 70 (1998) 4974.
[2] B. He, N. Tait, F. Regnier, Anal. Chem. 70 (1998) 3790.
[3] M. Kuschel, O. Mueller, Bioforum Int. 5 (2001) 290.
[4] S.V. Ermakov, S.C. Jacobson, J.M. Ramsey, Anal. Chem. 72 (2000) 3512.
[5] C. Yu, M. Xu, F. Svec, J.M.J. Jean, J. Polym. Sci. 40 (2002) 755.
[6] B.E. Slentz, N.A. Penner, F. Regnier, J. Chromatogr. A 948 (2002) 225.
[7] S. Wang, X. Zhang, F. Regnier, J. Chromatogr. A 949 (2002) 153.
[8] F. Hsieh, H.Q. Wang, C. Elicone, J. Mark, S.A. Martin, F. Regnier, Anal. Chem. 68 (1996) 455.
[9] F. Regnier, A. Amini, A. Chakraborty, M. Geng, J. Ji, L. Riggs, C. Sioma, S. Wang, X. Zhang, LC–GC 19 (2001) 200.
[10] F. Regnier, L. Riggs, R. Zhang, L. Xiong, P. Liu, A. Chakraborty, E. Seeley, C. Sioma, R.T. Thompson, J.Mass Spectrom. 37 (2002) 133.
[11] F. Le Naour, D.E. Misek, M.C. Krause, L. Deneux, T.J. Giordano, S. Scholl, S.M. Hanash, Clin. Cancer Res. 7 (2001) 3328.
[12] R. Aebersold, S.P. Gygi, T.J. Griffin, D.K. Han, M.J. Yelle, Am. Genomic/Proteomic Technol. 22 (2001) 26.
[13] R. Aebersold, D.R. Goodlett, Mass Spectrometry in Proteomics, Chem. Rev. 101 (2001) 269.
[14] M. Belew, J. Porath, J. Chromatogr. 516 (1990) 333.
[15] M. Geng, X. Zhang, M. Bina, F. Regnier, J. Chromatogr. A 752 (2001) 293.
[16] L. Riggs, C. Sioma, F. Regnier, J. Chromatogr. A 924 (2001) 359.
[17] J. Li, T. LeRiche, T. Tremblay, C. Wang, E. Bonneil, D.J. Harrison, P. Thibault, Mol. Cell. Proteomics 1 (2002) 157.
[18] B.E. Slentz, N.A. Penner, E. Lugowska, F. Regnier, Electrophoresis 22 (2001) 3736.
[19] G.R. Bogart, D.E. Leyden, T.M. Wade, W. Schafer, P.W. Carr, J. Chromatogr. 483 (1989) 209.
[20] F.B. Anspach, J. Chromatogr. A 672 (1994) 35.
[21] A.K. Singh, A.W. Flounders, J.V. Volponi, C.S. Ashley, K. Wally, J.S. Schoeniger, Biosens. Bioelectron. 14 (1999) 703.
[22] POROS 20 AL Media—Immobilizing Ligand for Perfusion Immuno Assays, PerSeptive Biosystems, 1996.
[23] S.C. Jacobson, L.B. Koutny, R. Hergenroeder, A.W. Moore, J.M. Ramsey, Anal. Chem. 66 (1994) 3472.
[24] B. He, B.J. Burke, X. Zhang, R. Zhang, F. Regnier, Anal. Chem. (2001) 1942.
[25] B.J. Burke, F.E. Regnier, Electrophoresis 22 (2001) 3744.
[26] L. Xiong, F. Regnier, J. Chromatogr. A 924 (2001) 165.
[27] K. Haupt, F. Roy, M.A. Vijayalakshmi, Anal. Biochem. 234 (1996) 149.
[28] K.Y. Jiang, O. Pitiot, M. Anissimova, H. Adenier, M.A. Vijayalakshmi, Biochim. Biophys. Acta 1433 (1999) 198.
[29] B. He, L. Tan, F. Regnier, Anal. Chem. 71 (1999) 1464.

[30] C. Wang, R. Oleschuk, F. Ouchen, J. Li, P. Thibault, D.J. Harrison, Rapid Commun. Mass Spectrom. 14 (2000) 1377.
[31] Q. Xue, F. Foret, Y.M. Dunajevskiy, B.L. Karger, Rapid Commun. Mass Spectrom. 11 (1997) 1253.
[32] S. Wang, F. Regnier, J. Chromatogr. A 913 (2001) 429.
[33] J.H. Chan, A.T. Timprman, D. Qin, R. Aebersold, Anal. Chem. 71 (1999) 4437.
[34] J.S. Kim, D.R. Knapp, Electrophoresis 22 (2001) 3993.