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Short communication

Capillary electrochromatography of peptides in a microfabricated system

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Abstract

Reversed-phase liquid chromatography of tryptic peptides is shown in the capillary electrochromatography mode using microfabricated columns. Although selectivity is different, a mixture of tryptic peptides from ovalbumin appears to be as easily separated in the CEC as HPLC mode. The major difference between a separation in the microfabricated CEC column and conventional separations in the HPLC mode is that separations are more readily achieved in the isocratic mode in the lower surface area microfabricated CEC columns.

Keywords: Electrochromatography; Capillary columns; Peptides

1. Introduction

The literature is expanding rapidly on microfabricated analytical systems that include a separation component [1–5]. In most cases, the separation engine in these has been some form of capillary electrophoresis (CE). The great advantage of CE on chips is that separations may be accomplished with electrical potential alone. Capillary electrochromatography (CEC) is a similar case [6,7]. Application of potential across a column packed with a silica based sorbent will induce a flow of liquid due to electroosmosis [8], also known as electroendoosmosis. Electroosmotic flow (EOF) results from the formation of an ionic double layer at charged surfaces and the movement of this layer and the surrounding solvent under an electrical potential. EOF provides the requisite flow to achieve chromatographic separations in a CEC system.

The problem with incorporating CEC into microfabricated systems is that it is difficult to pack columns on a chip. Initial studies reported the use of columns of 100×20 μm rectangular cross section with an etched filter at the outlet to retain sorbent particles [9]. No provision was made to physically retain the packing material at the inlet. Obviously there will be substantial packing heterogeneity at the corners of a rectangular column. Two alternatives to packed columns have been examined. One supported the stationary phase directly on the channel walls [10,11]. The column was etched into a glass substrate, making it easy to attach organosilane stationary phases to the silanol rich walls. A second alternative is to mimic the packed bed by etching an array of support particles into a quartz substrate [11]. Columns based on an array of collocate monolith support structures (COMOSS) have several advantages. Among the more important are that (i) the COMOSS are all formed simultaneously during the etching process, (ii) the support structures are defined in size and positioned in the column to within 0.1 μm, and (iii) they are bonded to the column walls to keep them from shifting during use. Addi-

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tionally, (i) channel dimensions in etched columns are independent of any packing process, (ii) the channels are dimensionally homogenous, (iii) channel width can be varied independent of the size and shape of the support structures, and (iv) particle distribution is extremely uniform. A potential limitation of all the etched columns is that their phase ratio is an order of magnitude, or more, lower than columns packed with porous particles.

Unfortunately, there are few applications of CEC on chips to problems in the life sciences. For example, tryptic mapping by reversed-phase liquid chromatography is now widely used in protein characterization [12] but it is not well documented in the CEC mode. There is also great interest today in proteomics [13–15] and the use of tryptic digests to characterize large numbers of proteins from many samples [16]. The question is how large numbers of tryptic digests will be analyzed and whether CEC could play a role. It is possible that one solution to the throughput problem could be to use parallel CEC columns in chip based systems. But before parallel processing systems on chips are developed, it is prudent to demonstrate that peptide separations on chips are possible. The efficacy of CEC in tryptic mapping ovalbumin is reported in this paper.

2. Materials and methods

2.1. Reagents

Ovalbumin, trypsin, trifluoroacetic acid (TFA), potassium phosphate, calcium chloride, tris(hydroxymethyl)aminomethane (Tris) and Tris–HCl were obtained from Sigma (St. Louis, MO, USA). PerSeptive Biosystems (Framingham, MA, USA) supplied the PepMap C_{18} column.

2.2. Equipment

All high-performance liquid chromatography (HPLC) experiments were carried out with a BioCAD Work Station from PerSeptive Biosystems. CEC and laser induced fluorescence detection were achieved with an instrument system assembled from available components as previously described [11].

2.3. Preparation of the CEC column

The C_{18} COMOSS column used in these experiments was prepared as previously described [11].

2.4. Tryptic digestion of ovalbumin

Ovalbumin was digested at a concentration of 1 mg/ml using a 50:1 ovalbumin to trypsin ratio in a 24-h reaction at pH 8.0 in a 10 m*M* $CaCl_2$–50 m*M* Tris buffer.

2.5. Fluorescein-5-isothiocyanate (FITC) labeling

FITC was prepared as a 1 m*M* solution in acetone with a trace of pyridine. Trypsin-digested ovalbumin was prepared at a concentration of 1 mg/ml and added to the FITC labeling solution in a molar excess of 3 to 1. The labeling reaction was carried out at room temperature for 4 h and the labeled peptides used within 24 h of derivatization.

2.6. HPLC separations

The ovalbumin tryptic digest was resolved in HPLC by gradient elution of a 250×4.6 mm PepMap C_{18} column at 1 ml/min with a mobile phase ranging from 5% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) to 75% ACN in 0.1% TFA in 60 min.

2.7. CEC separations

Peptides were eluted from the C_{18} COMOSS column (1.5 μm wide and 10 μm deep) using a potential of 770 V/cm (10 μA) and a mixture of 10 m*M* potassium phosphate buffer (pH 9)–acetonitrile (3:1, v/v) at room temperature. The effective COMOSS column length was 4.5 cm.

2.8. Capillary zone electrophoresis (CZE)

Conditions for CZE were identical to those used for CEC.

3. Results and discussion

It is seen (Fig. 1) that a tryptic digest of ovalbumin can be resolved by HPLC using a C_{18} reversed-phase liquid chromatography (RPLC) column with a mobile phase gradient ranging from 5% ACN in 0.1% TFA to 75% ACN in 0.1% TFA. Detection in this case was achieved by absorbance monitoring at 215 nm. Theoretically, there should be 36 peptides in this mixture based on the specificity of trypsin and the sequence of ovalbumin. Less than the theoretical number of peptides is frequently seen in tryptic digests for several reasons. One is that very small, hydrophilic peptides elute from RPLC columns unretained. Another is that similar peptides co-elute.

A similar separation of this tryptic digest in the CEC mode (Fig. 2) on a C_{18} COMOSS column and in the CZE mode (Fig. 3) using the same column without a C_{18} bonded phase show that the CEC separation is unique. Although the number of peaks in the two chromatograms (Figs. 1 and 2) is roughly the same, there are differences in the samples, conditions, and results that should be noted. One is that detection in the CEC mode was achieved using laser induced fluorescence with FITC-tagged peptides. Because the FITC tag adds substantial hydrophobicity to peptides, it is to be expected that

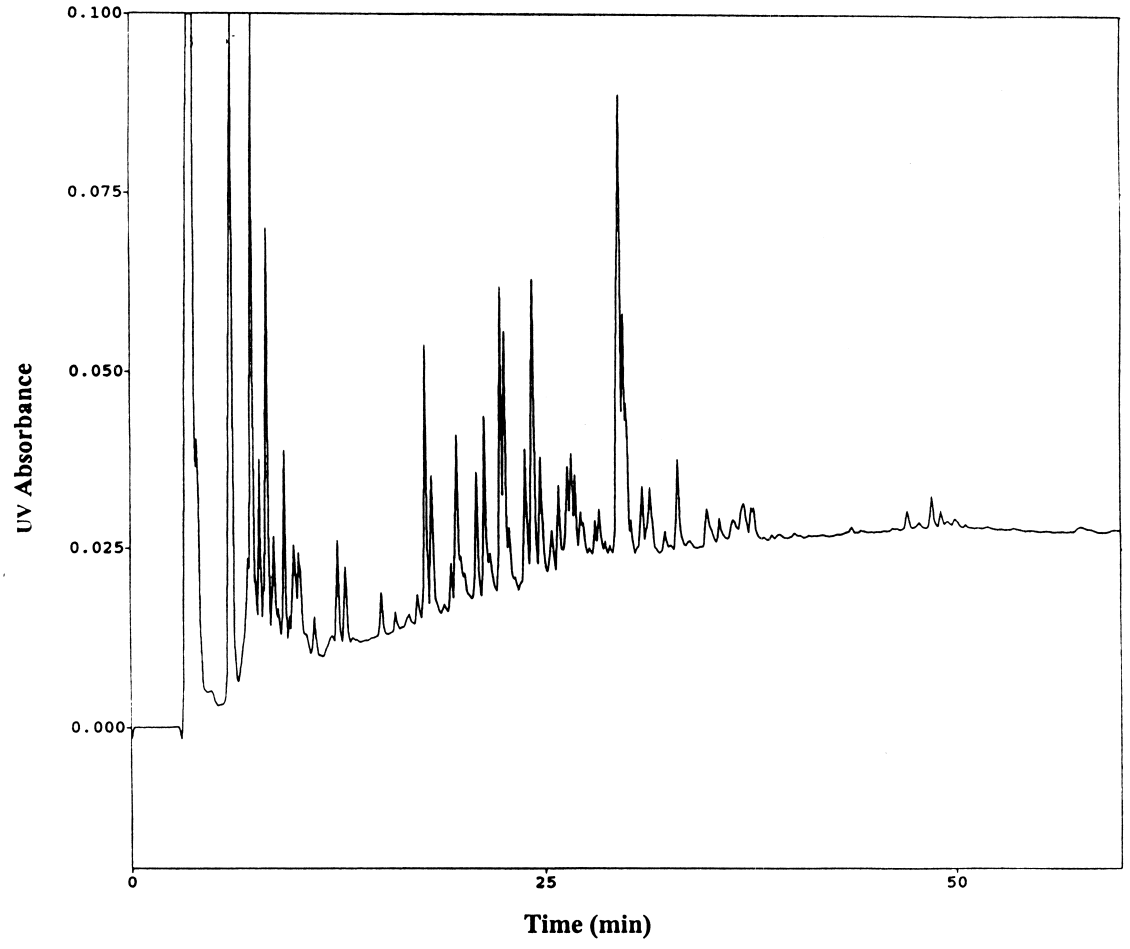

Fig. 1. A reversed-phase liquid chromatogram of a tryptic digest of ovalbumin with the column operated in the HPLC mode. The digest was resolved by gradient elution of a 250×4.6 mm PepMap C_{18} column at 1 ml/min with a mobile phases ranging from 5% acetonitrile (ACN) in 0.1% trifluoracetic acid (TFA) to 75% ACN in 0.1% TFA in 60 min.

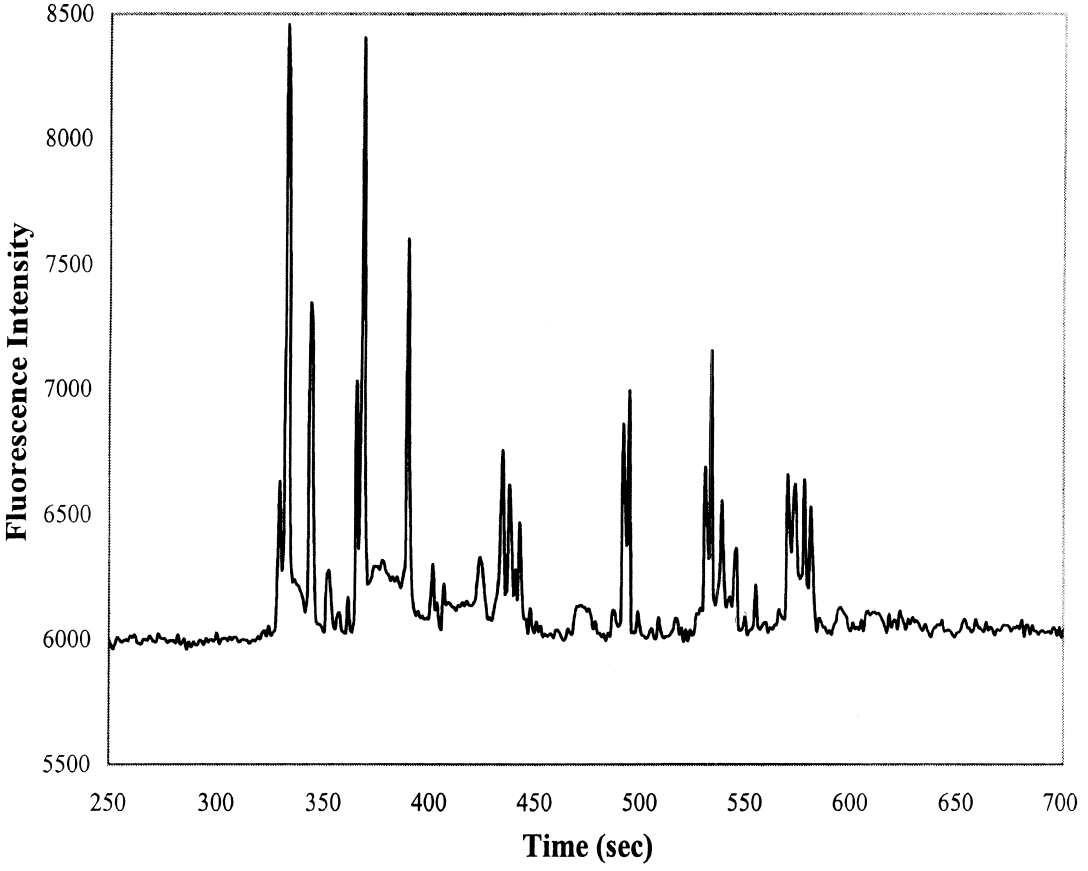

Fig. 2. A reversed-phase chromatogram of the tryptic digest of ovalbumin with a C_{18} COMOSS column operated in the CEC mode. Analytes were eluted from the column (1.5 μm wide and 10 μm deep) at a potential of 770 V/cm (10 μA) with a 10 m*M* potassium phosphate buffer (pH 9)–acetonitrile (3:1, v/v) at room temperature. The effective COMOSS column length was 4.5 cm.

selectivity will be different in these two cases. A second difference is that the separation in the CEC mode was executed at pH 9.0 because FITC does not fluoresce well at acidic pH. Separating at basic instead of acidic conditions alters selectivity further. The third major difference was that the tryptic digest of ovalbumin elutes from the C_{18} COMOSS column isocratically. Obviously, the C_{18} COMOSS column is far less retentive. It is expected that FITC tagging, changing mobile phase pH, and contributions from the electrophoretic mobility of analytes in CEC will make major changes in retention, but not to the extent that elution could be achieved isocratically.

It could be argued that perhaps the stationary phase density is so low in the C_{18} COMOSS column that the peptides are separating by electrophoretic mobility alone. This is seen not to be the case in Fig. 3 where CZE of the tryptic digest on an uncoated COMOSS column at pH 9.0 is inferior to that achieved on the C_{18} coated column. Resolution of the tryptic digest at pH 9 is very poor by CZE alone, although a better separation of the given peptide mixture could be achieved by CZE if the separation conditions are optimized. It is not possible that the separation seen in Fig. 2 is from CZE.

The more likely explanation for the very large decrease in retentivity of the CEC column is the very large difference in phase ratio. It has been estimated that the surface area-to-volume ratio in a COMOSS column is approximately equal to that of a column packed with 4–5 μm non-porous particles [17]. Although the surface area to volume ratio of the

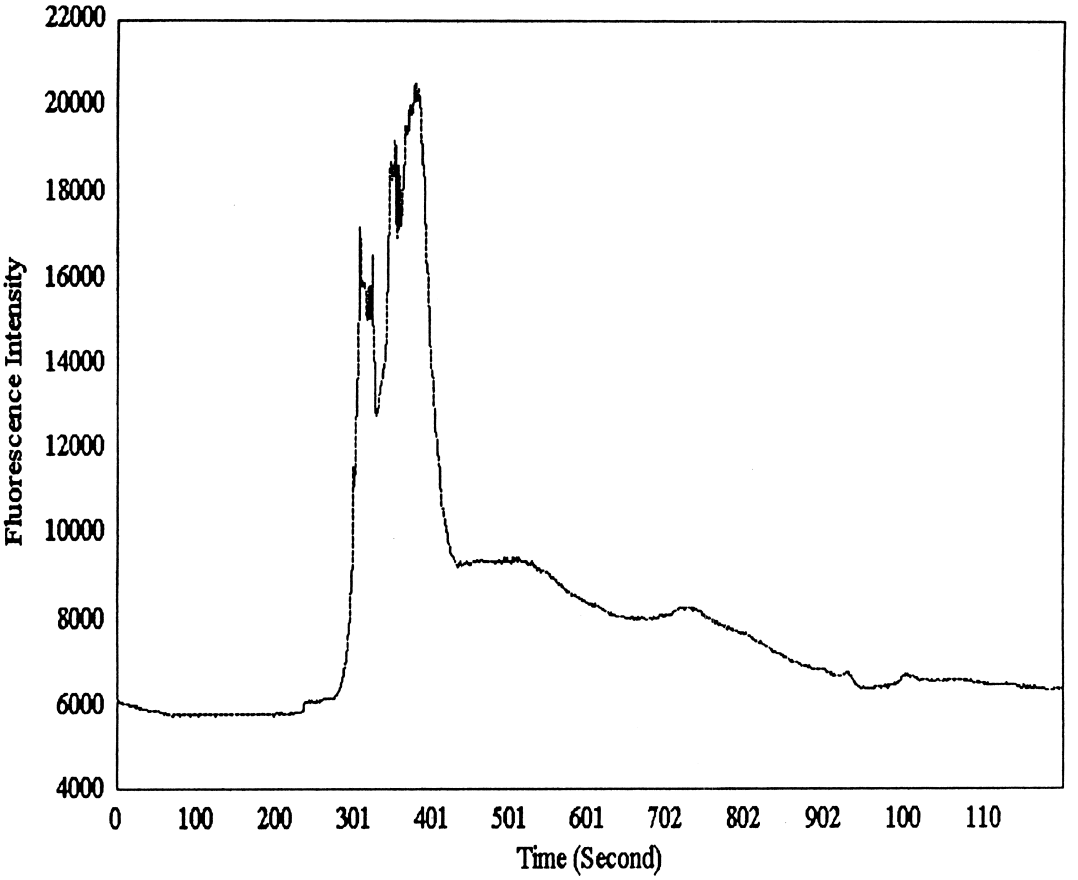

Fig. 3. CZE of the tryptic digest on an uncoated COMOSS column (1.5 μm wide and 10 μm deep). Elution was achieved using a potential of 770 V/cm (10 μA) with a 10 m*M* potassium phosphate buffer (pH 9) at room temperature. The effective COMOSS column length was 4.5 cm.

porous particle HPLC column was not available, the phase ratio of porous packings is generally 100-times that of non-porous particles. Because capacity factor is directly proportional to the phase ratio, it is easily seen why peptides elute so easily from the COMOSS column.

4. Conclusions

It is concluded from these preliminary studies that CEC of peptides in microfabricated columns is possible and that peak capacity is comparable to that of HPLC. Moreover, CEC in low-phase-ratio microfabricated columns has the major advantage that mixtures can be resolved isocratically as opposed to the gradient elution mode in HPLC. This makes the chromatographic process much simpler, especially in chips where the need for a gradient generator is circumvented. Finally, it is concluded that further development of parallel processing CEC systems on chips for peptide separations is justified.

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References

[1] S. Pace, US Pat., 4 908 112 (1990).
[2] K.R. Mitchelson, J. Cheng, L.J. Kricka, Trends Biotechnol. 15 (11) (1997) 448.
[3] L.B. Koutny, D. Schmalzing, T.A. Taylor, M. Fuchs, Anal. Chem. 68 (1996) 18.
[4] S.C. Jacobson, M. Ramsey, Anal. Chem. 68 (1996) 720.
[5] T. Woolley, D. Hadley, P. Landre, A.J. deMello, R.A. Mathies, M.A. Northrup, Anal. Chem. 68 (1996) 4081.
[6] M.M. Robson, M.G. Cikalo, P. Myers, M.R. Euerby, K.D. Bartle, J. Microcol. Sep. 9 (1997) 357.
[7] L.A. Colon, Y. Guo, A. Fermier, Anal. Chem. 69 (1997) 461.
[8] A.J.W. Jorgenson, K.D. Lukacs, Anal. Chem. 53 (1981) 1298.
[9] G. Ocvirk, E. Verpoorte, A. Manz, M. Grasserbauer, H.M. Widmer, Anal. Methods Instrum. 2 (1995) 74.
[10] S.C. Jacobson, R. Hergenroder, L.B. Koutny, J.M. Ramsey, Anal. Chem. 66 (1994) 2369.
[11] B. He, N. Tait, F.E. Regnier, Anal. Chem. 70 (1998) 3790.
[12] J. Lei, D. Chen, F.E. Regnier, J. Chromatogr. A 808 (1998) 121.
[13] I. Humphery-Smith, W. Blackstock, J. Protein Chem. 16 (5) (1997) 537.
[14] M.R. Wilkins, J.-C. Sanchez, A.A. Gooley, R.D. Appel, I. Humphery-Smith, D.F. Hochstraser, K.L. Williams, Biotechnol. Genet. Eng. Rev. 13 (1995) 19.
[15] P. James, Biochem. Biophys. Res. Comm. 231 (1997) 1.
[16] J. Houthaeve, H. Gausephohl, M. Mann, L. Ashman, FEBS Lett. 376 (1995) 91.
[17] B. He, Ph.D. Thesis, Purdue University, West Lafayette, IN, 1998.