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Capillary electrochromatography of peptides on microfabricated poly(dimethylsiloxane) chips modified by cerium(IV)-catalyzed polymerization

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Abstract

Vinylsulfonic acid, acrylic acid, 2-acrylamido-2-methylpropanesulfonic acid (AMPS), 4-styrenesulfonic acid, and stearyl methacrylate were used for successful modification of the surface of poly(dimethylsiloxane) (PDMS) by cerium(IV) catalyzed polymerization on microfabricated collocated monolith support structures microchips. Reproducible and stable coatings were obtained allowing highly efficient separations of a peptide mixture with RSD for retention times below 2.6%. AMPS-coated PDMS channels were shown to give a reproducible separation of a synthetic peptide mixture for over a month. Subsequent modification of microchip channels by AMPS and methoxydimethyloctadecylsilane allowed selective separation of complex bovine serum albumin digest with high reproducibility, and efficiency of about 620 000 plates/m.

Keywords: Electrochromatography; Chip technology; Peptides; Poly(dimethylsiloxane)

1. Introduction

There is currently substantial interest in liquid chromatography systems on a chip. At present, the capillary electrochromatography (CEC) approach has been more successful than HPLC because it is still very difficult to build high-pressure pumps on a chip [1–9]. CEC is a hybrid between capillary electrophoresis (CE) and microcolumn HPLC that combines advantages of both techniques. An advantage of CEC over CE is the possibility that both charged and uncharged analytes may be separated with high selectivity and efficiency in a relatively short time. CEC is very similar to HPLC except that in CEC: (i) the mobile phase is driven by voltage alone, (ii) there may be an electrophoretic mobility component to separations, and (iii) the flow profile in CEC produces less band spreading than in HPLC.

The greatest problem in CEC is preparation of separation columns where the stationary phase needs to contain both selectivity for separation, and also charged groups for generation of sufficient, stable, and reproducible electroosmotic flow (EOF). After complaining and striving for many years to remove charge from reversed-phase columns, chromatographers must again cope with charged stationary phases in CEC.

The vast majority of efforts to produce CEC columns mimic standard HPLC column technology where the object is to prepare columns packed with particles of 1.5–5 μm in size with different functionalities [5,7,10]. However, approaches designed for conventional CEC have proved difficult to imple-

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ment on the microchip scale. Clearly, new ways are needed for the preparation of liquid chromatography columns on chips.

Three different approaches to the fabrication of liquid chromatography columns on chips have been described [11–21]. One is the classical protocol of slurry packing particles into the chromatography channel [16,19,20]. The major problem in this case is poor efficiency due to nonhomogeneity of packing. In addition, there is a necessity to design frits into the chips to retain particles. Another approach is to form a continuous, porous bed of support in capillaries on a chip by in situ polymerization of organic monomers [22–25]. These ''monolithic'' columns have the great advantage that the support and stationary phase for the whole column are formed simultaneously without packing. Finally, there is a direct fabrication strategy.

The direct fabrication alternative is based on the use of micromachining or photolithography to produce all the channels and support surfaces in a column [11]. Bundles of intermittently crossing channels of identical geometry are formed in the chip substrate to direct liquid through columns in much the same way as in conventional chromatography columns. The most common of these columns has the channels crossing at right angles to form rows of support structures along the length of the column. These collocate monolith support structures (COMOSS) have several advantages. Among the most important are that the COMOSS are all formed simultaneously, the support structures are defined in size and positioned in the column to within 0.1 μm, and they are bonded to the chip substrate to keep them from shifting during use. Additionally, channels are homogenous in dimensions and COMOSS size distribution is extremely uniform [13].

The possibility of using COMOSS chips made from poly(dimethylsiloxane) (PDMS) was recently demonstrated [26]. After oxidation or exposure to sodium hydroxide, the PDMS surface is rich in silanol groups [27]. This allowed the channel walls to be modified with an either octyl- or octadecylsilane stationary phase and used for the separation of tryptic digested proteins and simple organic compounds [26]. These stationary phases gave high efficiency and good resolution of peptides and small organic compounds in columns of 3.9 cm length [26].

A problem with CEC columns based on C_8 and C_{18} stationary phases alone is that EOF depends on dissociation of residual surface silanols. These silanol groups are generally of low density, their dissociation is pH-dependent, they are not uniformly distributed, and the number of surface silanols tends to decrease as organosilane loading increases. Thus, EOF is low and often non-reproducible. Addition of sodium dodecylsulfate (SDS) to the mobile phase enhances EOF in reversed-phase columns, but leads to a sharp increase in current [26]. It is therefore necessary to operate columns at low separation voltages to avoid extensive Joule heating that leads to solvent vaporization along with other negative effects. The alternative to SDS is to have charge present in the stationary phase. This is generally accomplished by the use of a charged organosilane that is also hydrophobic. Unfortunately, the number of charged silanes that are sufficiently hydrophobic to be used for reversed-phase production is limited.

Another approach would be to uncouple the addition of charged and hydrophobic groups to the column surface. The presence of charged polymer on COMOSS surfaces would dramatically increase EOF, potentially without precluding the attachment of a hydrophobic stationary phase. Also, there is a long history of attaching charged polymers to chromatography supports that could be exploited. It has been previously demonstrated that cerium(IV) initiated grafting of polymer chains on to the internal surface of porous beads affords an excellent separation medium for biopolymers [28]. Such modification would be advantageous because a wide variety of commercially available, low cost, reagents could be used as monomers in a production of stationary phases. For the grafting and subsequent polymerization at surfaces to occur with Ce(IV), the surface must contain OH-groups. Although PDMS surfaces originally contain no alcohols, they can be activated in a plasma oxidizer. Ce(IV) catalyzed grafting has been widely used in the modification of natural and synthetic polymers [28], but it has never been used to modify silanol surfaces in PDMS [29,30].

The objective of this paper was to study the possibility that oxidized PDMS COMOSS columns could be modified by cerium(IV) catalyzed polymerization to prepare highly efficient media for the CEC separation of peptide mixtures.

2. Experimental

2.1. Materials

Sylgard 184 PDMS base polymer and curing agent were purchased from Dow Corning (Midland, MI, USA). Fluorescein sodium salt, methoxydimethyloctadecylsilane, stearyl methacrylate, vinylsulfonic acid, polyacrylic acid, 4-styrenesulfonic acid, Gly–Tyr peptide, fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) and 2-acrylamido-2-methylpropanesulfonic acid (AMPS) were purchased from Sigma–Aldrich (St Louis, MO, USA). Sodium hydrogencarbonate, potassium carbonate, and acetonitrile (ACN) were purchased from Malinckrodt (Paris, KY, USA). Propanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium cerium(IV) nitrate was purchased from Lancaster (Pelham, NH, USA). Dodecyl sulfate, sodium salt was purchased from ACROS (Geel, Belgium) and H–Gly–Phe–Glu–Lys–OH peptide from BACHEM Bioscience (King of Prussia, PA, USA). Fluorescein-5-isothiocyanate (FITC isomer I) was purchased from Molecular Probes (Eugene, OR, USA).

2.2. Modification of PDMS chips

PDMS separation columns were molded from a positive photoresist design master as previously described [26]. The scheme of the column used in this paper with dimensions is presented in Fig. 1. After oxidation, a PDMS mold and cover slab were brought into contact to make an irreversible seal. Reaction mixtures, which were comprised of 50 mg of monomer, 5 mg of ammonium cerium nitrate, 0.2 ml of 1 *M* nitric acid in 5 ml of solvent, which was water for AMPS, vinyl sulfonic acid, and acrylic acid, water–ethanol (1:4) in case of 4-styrenesulfonic acid and ethanol for stearyl methacrylate–AMPS, were immediately placed into wells of the chip allowing capillary action to fill the separation column. Reaction proceeded for 30–60 min at room temperature and then 1 m*M* carbonate buffer (pH 9) was electroosmotically pumped through the channels for 15 min. Structures of grafted polymers used in this paper are presented in Fig. 2.

A C_{18}–AMPS column was produced by modification of an AMPS column with methoxydimethyloctadecylsilane, dissolved in 2.5% SDS in water [26], that was electroosmotically pumped through the channels and incubated for 15 min. This was followed by washing with 1 m*M* carbonate buffer for 15 min.

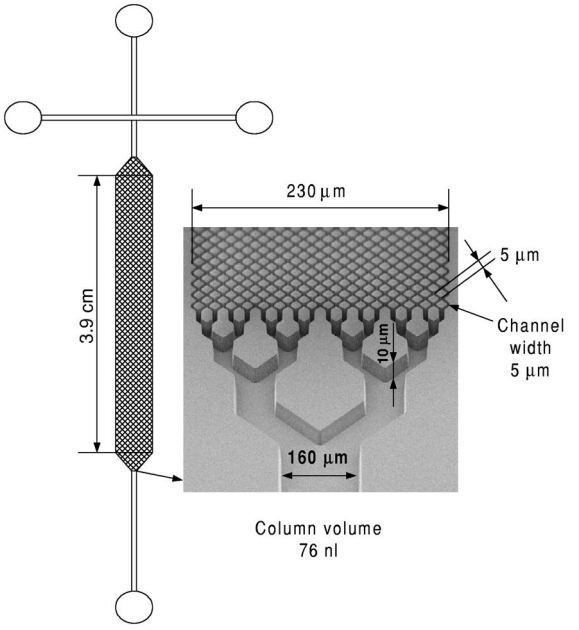


Fig. 1. Scheme of a COMOSS separation column used in this study.

2.3. Instrumentation

Visualization of fluidic movement in channels was realized using a Nikon Inverted Eclipse TE-300 optical microscope via a TE-FM confocal-fluorescence system with fluorescent samples, 1×10^{-4} *M*, as previously described [31]. Data collection was on a laboratory-built epi-fluorescence system as previously described [26].

2.4. Injection of samples

Electrokinetic injections were made using the gated injection as described by Ramsey et al. [32] except injection voltage was kept constant at 1000 V. Diffusion injection followed the same procedure, except injection voltage was kept at 0 V. Time of injections was varied from 0.25 to 90 s.

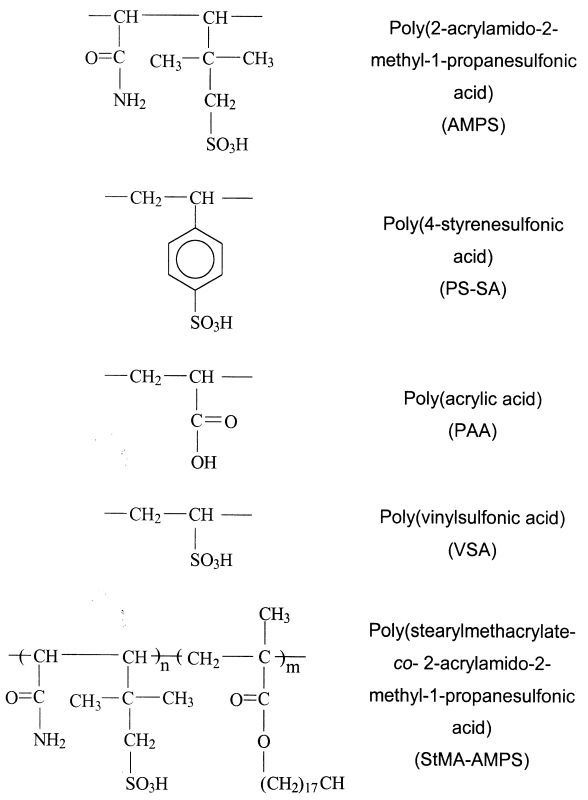


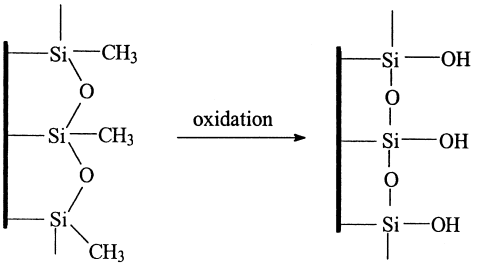
Fig. 2. Structures of grafted polymers and abbreviations.

3. Results and discussion

3.1. AMPS-modified columns

It was shown recently that the surface properties of unmodified PDMS are constantly changing [26], thus causing differing selectivities and retention times of separations. Also, due to the silanol groups producing the charge on a PDMS surface, EOF is greatly reduced at acidic pH. So, the search for stationary phases that can provide both stable EOF over a wide pH-range and high selectivity of separation is of great importance. AMPS has often been used as an EOF enhancing additive in the production of polymer monoliths for CEC [22,33,34]. It can even be grafted to the surface of polymethacrylate [26]. The presence of sulfonic acid groups thus introduced provides a stable surface charge and constant EOF over a wide pH-range.

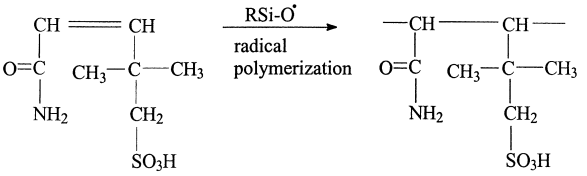
A PDMS replica of a COMOSS CEC column was formed via the molding procedure previously described [26]. Following oxidation and sealing of a PDMS cover slab to the molded COMOSS column, free silanol groups at the surface of the resulting column were available for further modification.



The modification process on the surface is activated perhaps according to simplified mechanism shown below that has been proposed in the literature [35]:

$$\mathrm{Ce(IV) + RCH_2OH \rightarrow Ce(III) + H^+ + RCH_2O^{\cdot}}$$

The reaction appears to proceed in the presence of silanol groups as it would with C–OH groups. Upon activation, the silanol radicals initiate polymerization with the double bonds in an AMPS molecule:



Radical polymerization produces a crosslinked polymer that is attached to the surface only at the first moment of reaction, through a Si–O–C bridge. It is well known that this bridge is easily hydrolyzed by water reforming a silanol group. As a result, this insoluble polymer does not stay chemically attached, but it is physically adsorbed to the surface. Low concentrations of monomer and catalyzing agent were reacted at room temperature to decrease the rate of polymerization and obtain a thin, permeable polymer layer allowing, if desired, subsequent modification of the PDMS surface.

Efficiencies of over 300 000 plates/m were realized in the separation of FITC-labeled peptides with the AMPS modified COMOSS columns. The reproducibility of retention times obtained on the same day was relatively high, having a relative standard deviation (RSD)$<$2.2%. The AMPS column was

also found to be stable and reproducible for a long period of time. Separations of the same mixture of peptides during a month were almost identical (Fig. 3). There was a small drift toward a reduction of retention time on the order of 3 s for the peak with highest retention time. This indicates that AMPS is not leaching from the column. RSD of retention times of peptides over all separations was 3.0–5.3%.

The first two peaks in the separation are H–Gly–Phe–Glu–Lys–OH, the third peak is unreacted FITC, while the last peak is H–Gly–Try–OH. In this case we have heavier peptides eluting before the peptide with lower mass, moreover, two peaks correspond to the H–Gly–Phe–Glu–Lys–OH peptide. Due to the presence of Lys in H–Gly–Phe–Glu–Lys–OH, the peptide was doubly labeled as confirmed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The single- and double-labeled peptides separate because of the extra negative charge from the FITC molecule. Because H–Gly–Phe–Glu–Lys–OH has an extra negative charge from dissociation of carboxylic group of Glu, it moves more quickly than the lighter H–Gly–Try–OH.

3.2. Modification with poly(styrenesulfonic acid), polyacrylic acid and poly(vinylsulfonic acid)

According to the mechanism of Ce(IV) catalyzed polymerization, its possible to use a large variety of commercially available monomers to modify oxidized PDMS COMOSS channels. Among the most conventional are vinylsulfonic, styrenesulfonic, and acrylic acids, with the latter containing a pH-dependent acidic group. The acrylic acid and vinylsulfonic acid monomers have similar structure, except for the type of acidic group. It was observed that the polyacrylic acid (PAA) phase produced a better separation of peptides than poly(vinylsulfonic acid) (VSA) (Fig. 4). Though both have comparatively high efficiency, PAA gave separations with relatively longer retention times, probably because of interactions of carboxylic acid groups with amino groups of peptides.

Poly(styrenesulfonic acid) (PS-SA) contains a hydrophobic phenyl group, thus it should separate the peptide mixture better than VSA and PAA, which was observed (Fig. 4). Although experiments

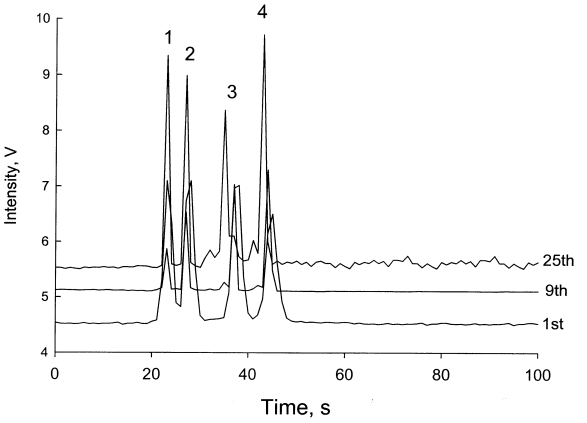


Fig. 3. Reproducibility of separation of FITC-labeled synthetic peptide mixture on AMPS-coated microchip. Mobile phase: 1 m*M* carbonate buffer (pH 9.0), 3000 V. (1) FITC–Gly–Phe–Glu–Lys(FITC)–OH; (2) FITC–Gly–Phe–Glu–Lys–OH; (3) FITC; (4) FITC–Gly–Tyr–OH.

showed that the efficiency of PS-SA was lower than VSA and PAA, VSA and PAA typically did not give base line separations of the test mixture.

It was concluded that monomers containing the sulfonic acid were beneficial for CEC separation of peptides on PDMS COMOSS chips because the EOF was more stable giving separations of higher quality. Among sulfonic acid group containing monomers, AMPS produced the most reproducible retention times over a long period of usage. To improve the

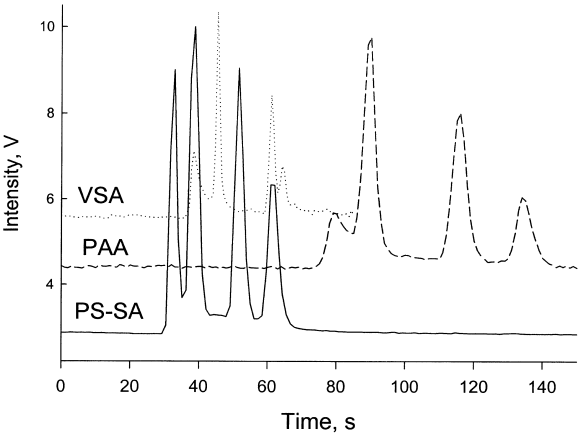
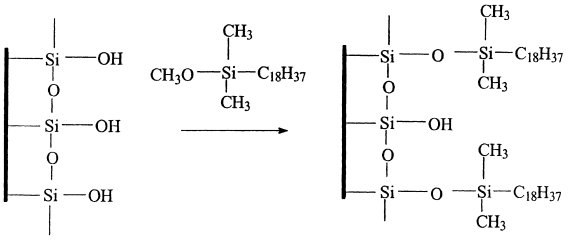


Fig. 4. Separation of FITC-labeled synthetic peptide mixture on microchip with various grafted stationary phases. Mobile phase: 1 m*M* carbonate buffer (pH 9.0), 1500 V. Sequence of peaks as in Fig. 2.

selectivity of AMPS-modified channels, a few attempts were made to introduce hydrophobic groups on to AMPS surface.

3.3. C_{18}–AMPS-modified columns

Octadecylsilane modified sorbents are the most popular for peptide separation in HPLC [36]. Because AMPS-modified PDMS was found to have stable EOF for a long period of time, it would be advantageous to create a stationary phase where the function of AMPS is to produce stable EOF, and C_{18} serves to introduce selectivity into separations. Toward this goal a two-step synthesis was developed that gave a mixed stationary phase containing negatively-charged AMPS groups and hydrophobic C_{18}-groups. C_{18}-silane reacts with free silanol groups that are still present on the surface of PDMS after polymerization of AMPS (or other monomers) is complete.



The polymer layer is thought to be permeable for silane molecules, and thus does not prevent silanization of the surface.

To compare AMPS and C_{18}–AMPS columns, selectivity coefficients between the last and first peaks of our test mixture were calculated. The selectivity coefficients were 1.80 and 1.94, respectively. Although the difference in coefficients is not very high, it is clear that the C_{18}–AMPS column possesses a higher retentivity. It should be mentioned that although there was little difference in retention times between C_{18}–AMPS and AMPS, the difference in efficiencies was remarkable: C_{18}–AMPS displayed double the efficiency of AMPS, showing greater than 600 000 plates/m.

A study of the dependence of separation efficiency on voltage (Fig. 5) showed that efficiency with peaks 1–4 steadily decreases with increasing voltage and concomitantly with flow-rate. The curve in Fig. 5

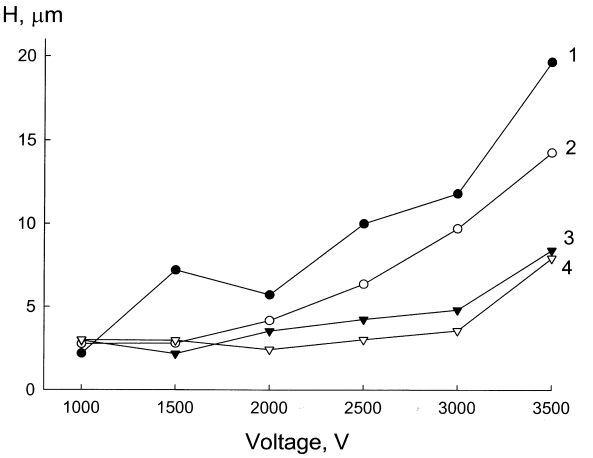


Fig. 5. Plot of plate height (H) versus voltage (V) for C_{18}–AMPS modified microchip. Mobile phase: 1 m*M* carbonate buffer (pH 9.0). (1) FITC–Gly–Phe–Glu–Lys (FITC)–OH; (2) FITC–Gly–Phe–Glu–Lys–OH; (3) FITC; (4) FITC–Gly–Tyr–OH.

corresponds to typical H versus voltage plots for CEC [19,37] and H versus flow-rate plots for HPLC. Obviously the decreasing efficiency with increasing voltage is a result of mass transfer limitations in the COMOSS column. This is most likely a mobile phase mass transfer effect. It was also observed that as the retention time increased from peak 1 to 4, so did efficiency.

A different route to synthesize an AMPS-based column with C_{18} groups is by co-polymerizing a mixture of AMPS and stearyl methacrylate (StMA–AMPS). This type of stationary phase gave separations of the same peptide mixture with larger retention times, but much lower efficiency (less than 60 000). Another problem was that clogging often took place during synthesis. Many columns had to be rejected in this approach. Optimization of the synthesis would perhaps overcome this problem.

3.4. Comparison of column performance

The performance and reproducibility of all columns made by the modification procedures described above were compared using the same peptide mixture and separation conditions. The maximum efficiency and RSD of retention times are presented in Table 1. AMPS modified columns showed the best efficiency among the stationary phases obtained by

Table 1
Efficiency of modified PDMS columns and reproducibility of retention times

Column	Efficiency, (plates/m)	t_R RSD (%)
AMPS	303 000	1.64 ($n=8$)
C_{18}–AMPS	620 000	0.68 ($n=3$)
PS-SA	44 000	2.58 ($n=3$)
VSA	224 000	0.69 ($n=4$)
PAA	115 000	1.77 ($n=2$)
StMA–AMPS	55 000	7.25 ($n=3$)
C_8	460 000	2.32 ($n=2$)
C_{18}	290 000	0.95 ($n=5$)

Ce(IV)-catalyzed polymerization. C_8 and C_{18} silane modified columns also gave high efficiency, but the combination of AMPS and octadecysilane resulted in its greatest increase. The reproducibility of retention times was good for all columns with an RSD$<$2.6%. The single exception was StMA–AMPS.

Addition of an organic modifier to mobile phase is usually considered as a way to differentiate between electrophoretic (CE) and partitioning (CEC) separation mechanisms. It was found that retention times for peptides from the test mixture on all columns studied in this paper decreased with addition of propanol to the running electrolyte. Thus, modified PDMS COMOSS microchip columns are performing in the CEC mode.

3.5. Modification of injection procedure

It is well known that in CE there is a problem with electrokinetic injection because of sampling bias. This is an even larger problem with peptides because of the wide variation in their net charge. Due to the difficulty of making pressure injections on microchips, the search for an alternative injection method is of great interest. Similar to electrokinetic injection based on electrophoretic mobility of analytes, it was rationalized that sample introduction could be made based on molecular diffusivity. When the molecular size of analytes is very similar, differences in molecular diffusivity will be much smaller than differences in electrophoretic mobility. Thus, it is expected that molecular diffusivity-based sample introduction will have less sampling bias than electrophoretic sample introduction. In this case, the amount of analyte introduced for analysis would depend on the time it resides in contact with the separation channel at zero potential.

Two chromatograms produced with electrokinetic and diffusion injection are presented in Fig. 6. First,

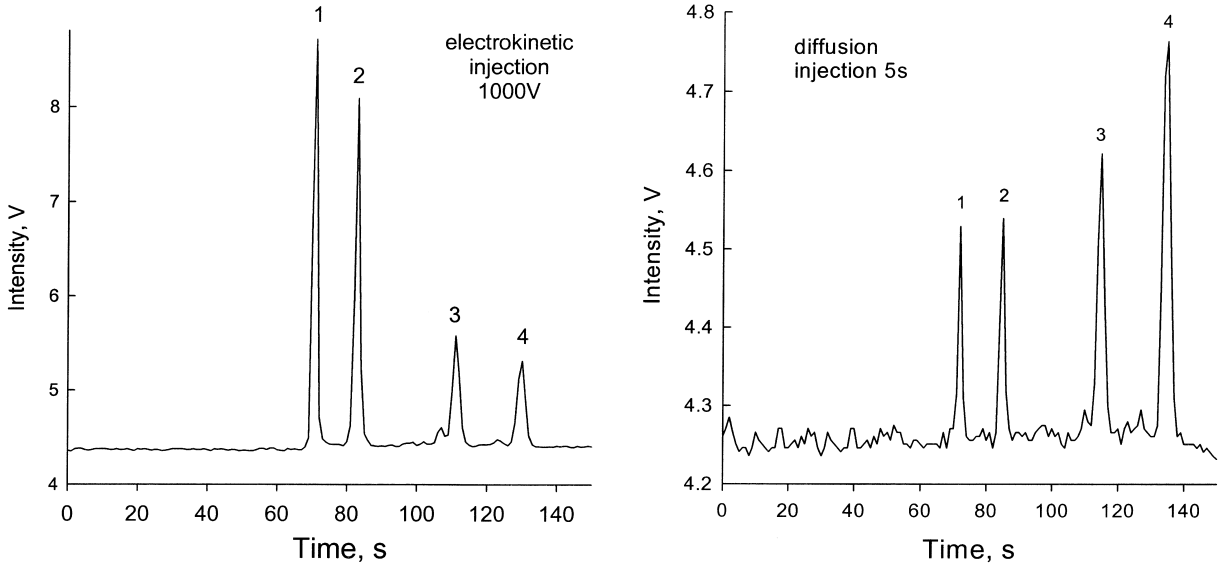


Fig. 6. Separation of FITC-labeled synthetic peptide mixture following electrokinetic and diffusion injection on C_{18}–AMPS modified microchip. Electrokinetic injection: 1000 V, 0.25 s. Diffusion injection time 5 s. Other conditions as in Fig. 2.

it is clear that different relative amounts of analytes were introduced into the separation channel. In the diffusion method, peaks 3 and 4 were injected at a relatively greater amount compared to electrokinetic injection because of their smaller size and thus higher diffusion rate. The distribution of sample components in the chromatogram produced with diffusion-based sample introduction was more like the relative concentration of components in the sample. Another advantage of diffusion injection is the ease of obtaining very small injection plugs. This is very important in separations on microchips. It should also be noted that smaller injection plugs from diffusion-based sample introduction gave higher efficiency than electrokinetic injection, i.e. 617 000 versus 245 000 plates/m, respectively for peak number 1.

3.6. Separation of FITC-labeled bovine serum albumin digest

The utility of the C_{18}–AMPS modified microchip with complex mixtures was tested using an FITC-labeled bovine serum albumin digest (Fig. 7). This mixture contained at least 12 FITC-labeled peptides as found by HPLC (data not shown). The microchip column showed good separation of this mixture within 5 min. Repetitive runs by either sample introduction method were reproducible, with nearly identical retention times (RSD$<$2.6%), peak shape, and intensity.

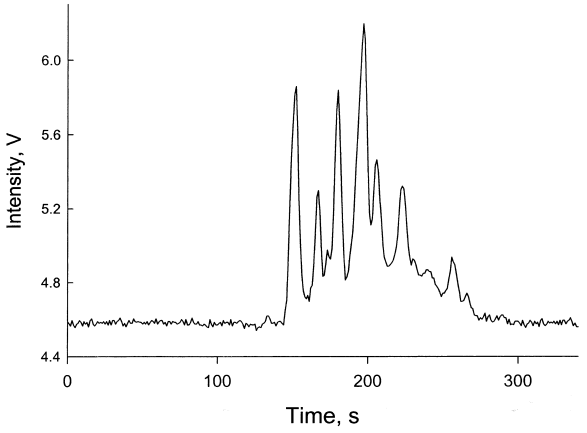


Fig. 7. Separation of peptides from FITC-bovine serum albumin digest on C_{18}–AMPS modified microchip, 1000 V. Mobile phase: 1 m*M* carbonate buffer (pH 9.0). Diffusion injection 60 s.

4. Conclusion

Five monomers were studied for modification of PDMS surfaces by Ce(IV)-catalyzed polymerization on COMOSS microchips. All were found to give a reproducible and stable coating of channel surfaces and to allow separation of peptide mixtures with RSD values for retention time below 2.6%. Plots of H versus voltage showed decreasing efficiency with increasing voltage, as is characteristic with CEC in general. AMPS-coated PDMS channels were shown to reproducibly separate peptide mixtures for over a month.

Subsequent modification of microchip channel by AMPS and octadecylsilane allowed selective separation of simple synthetic peptide mixtures and a more complex bovine serum albumin tryptic digest with high reproducibility and an efficiency of about 620 000 plates/m.

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