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Separation of mixtures of acidic and basic peptides at neutral pH

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Abstract

Mixtures of acidic and basic peptides composed of the phosphorylated and nonphosphorylated forms of peptide substrates for kinases and a phosphatase were separated by capillary electrophoresis (CE) in buffer conditions compatible with live mammalian cells. The separation of such mixtures was especially challenging given the high salt and neutral pH of the requisite physiologic buffers. Due to poor peak reproducibility in bare capillaries, several strategies were implemented to improve the electrophoretic separation of the peptide mixtures. Covalent coating of the capillary with the neutral polymer poly(dimethylacrylamide) (PDMA) resulted in a 2-fold improvement in the migration time RSD, but required the use of hydrodynamic flow to overcome the differing electrophoretic mobilities (μ_{eo}) of the peptides at neutral pH. This parabolic fluid flow diminished separation efficiency almost 5-fold. Polarity switching during the CE run was used to overcome the opposed μ_{eo}, but required the retention of hydrodynamic flow and consequent reduction in separation efficiency. The most efficient separations were seen with the use of covalently-linked, charged polymer coatings to maintain electroosmotic flow and to reduce wall interactions. Two such coatings were tested in the current study. Relative to the PDMA coating, an anionic poly(acrylate) improved the average migration time RSD of six peptides from 1.3 to 0.85% and average separation efficiency from 4.8 to 18.0 ($\cdot 10^4$ plates/m). Likewise, cationic poly([3-(methacryloylamino)propyl]-trimethylammonium) improved the average migration time RSD of eight peptides from 1.2 to 1.1% and average separation efficiency from 4.8 to 33.9 ($\cdot 10^4$ plates/m). These findings will be of value to the growing number of applications for analytical techniques utilizing CE for cellular analysis and biochemical studies.

Keywords: Hydrodynamic flow; Coated capillaries; Peptides; Enzymes

1. Introduction

CE has found utility for biochemical analyses, including the study of cellular analytes from individual cells [1–3]. Of relevance is the emergence of CE as a tool for the analysis of proteins and peptides including its use with mass spectroscopy [4,5]. Our own laboratory has developed a number of CE-based assays for measuring kinase activation in single, mammalian cells using peptide substrates for specific kinases [6]. This method relies on detecting and quantifying phosphorylated and nonphosphorylated peptides obtained from the cells. By virtue of the high separation efficiency of CE and the subattomole detection limits of laser-induced fluorescence detection, this method enables the measurement of multiple fluorescently-labeled peptides from a single cell containing nanomolar concentrations of the peptides. Thus, the number of kinases that can be assayed simultaneously is dependent on the separation performance of the CE analysis.

A significant drawback to the use of CE for the

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separation of mixtures of proteins and peptides is the degradation of separation performance caused by the adsorption of these bioanalytes to the surface of the capillary walls. This adsorption has been the subject of intense study, and it is apparent that the reductions in separation efficiency, sensitivity, and irreproducibility in migration times result from a variety of mechanisms [8–11]. In bare capillaries at neutral pH silanol groups (mean pK=6.3) are negatively charged so that extreme pH values of the running buffer or otherwise blocking these groups has been used to reduce adsorption [4,5,11,12]. However, these weakly acidic groups are necessary for the generation of the electroosmotic flow (EOF) whose presence is advantageous for reducing analysis times and for driving analytes, regardless of their charge, to the detection region [13].

A number of strategies have been utilized for improving the separation of peptide and protein mixtures and excellent reviews have been written on this subject in recent years [4,5,14–16]. A number of investigators have used buffers at the extremes of pH for peptide separations [17–22]. An alternative strategy has been the use of buffer additives to diminish peptide–wall interactions, for example polyethyleneimine or triethylamide [23–25]. Yet another method to control EOF and minimize peptide–wall interactions is the use of coatings to modify the capillary wall. A number of approaches can be taken to transform the capillary surfaces by physical adsorption, covalent bonding, or dynamic coating with a variety of agents [reviewed in 11,26,27].

Covalently-bonded polymer coatings have found widespread use in CE for peptide and protein separations. These polymers can possess a variety of chemical characteristics, and neutral, acidic, and basic polymers have all been employed [11,14,15]. Acrylamide-coating first described by Hjerten is particularly effective at resisting adsorption and is a popular method for increasing the separation efficiency of many different types of analytes [28–30]. Such neutral coatings result in suppressed EOF and are particularly useful where EOF is disadvantageous as in separations of molecules with small differences in mobilities [11]. These coatings are in general quite stable and have been used in the separations of a variety of basic or acidic proteins and peptides over a wide range of buffer conditions [31–35]. A number of cationic coatings have been used to reduce the adsorption of basic proteins while maintaining an anodal (reversed) EOF, while fewer reports have appeared in the literature on the use of anionic coatings [32,36–39]. Huang et al. have designed an anionic coating using 2-acrylamido-2-methylpropanesulfonic acid copolymerized in situ with acrylamide [38]. The resulting coated capillary demonstrated a constant cathodal EOF over a pH range of 3–10, and both acidic and basic peptides migrated in the same direction at pH 8.4. A similar approach has been used by Sun et al., but this coating was not tested for the separation of peptides or proteins [39].

While the methods described above result in excellent separation properties under appropriate conditions, a feature common to many of the techniques is that the electrophoresis buffers used are not compatible with biological cells. For studies such as ours of dynamic processes in living single cells, accurate measurements require that the sampling method has a subsecond temporal resolution [40]. In order to terminate intracellular biochemical reactions rapidly, cell disruption, analyte injection and electrophoresis must all take place without exchanging the physiologic buffer surrounding the cell with a traditional running buffer in the capillary. Mammalian cells exist in a high salt (~300 milliosmolar), neutral (pH 7.4) environment. Exposure of a cell to a typical low salt, extreme pH separation buffer prior to the time of the measurement will result in cellular stress or membrane damage. Likewise, many buffer additives used in peptide separations are toxic to living cells. Stress or membrane damage even for a few seconds prior to performing the measurement activate a number of cellular processes which utilize kinases and other enzymes, thus generating artifacts in the measurement of kinase activity [6,40–43]. For these reasons when measuring rapidly changing cellular phenomena with CE-based methods, it is often preferable for electrophoresis of cellular analytes to take place in a physiologic buffer in the absence of buffer additives. This requirement limits the conditions which can be varied for enhancing the separation of enzyme substrates obtained from living cells.

In the current paper, we sought to develop strategies to improve the CE separation of a mixture of

Table 1
Peptide kinase substrates used in the current study

Peptide name	Enzyme	Peptide sequence[a]	Net charge at pH 7.4	q/m ratio (1000×)
F-PKC	PKC	F-RFARKGSLRQKNV	+4	2.08
FP-PKC	PKC	F-RFARKGS(PO_3)LRQKNV	+2	1.0
F-PKA	PKA	F-KRREILSRRPSYR	+3	1.45
FP-PKA	PKA	F-KRREILS(PO_3)RRPSYR	+1	0.47
F-CaMKII	CaMKII	F-KKALHROETVDAL	0	0.0
FP-CaMKII	CaMKII	F-KKALHROET(PO_3)VDAL	−2	−1.03
FP-CAL	Calcineurin	F-DLDVPIPGRFDRRVS (PO_3) VAAE	−3	−1.18
F-CAL	Calcineurin	F-DLDVPIPGRFDRRVSVAAE	−1	−0.41

[a] F = Fluorescein (−1 charge at pH 7.4) [49].

acidic and basic peptides (see Table 1) in a physiologic buffer. The peptides are phosphorylated and nonphosphorylated pairs of peptide substrates for protein kinase C (PKC), protein kinase A (PKA), calcium-calmodulin activated kinase II (CaMKII), and the phosphatase calcineurin (CAL). A variety of polymeric capillary coatings were tested for their performance in separating these peptides having a range of charges at neutral pH (Table 1). The coatings included the neutral, hydrophilic coating poly(dimethyl acrylamide) (PDMA), a negative coating based on acrylic acid (acrylate), and a positive coating based on [3]-(methacryloylamino)propyl]-trimethylammonium chloride (MAPTA) (Fig. 1). The positively-charged MAPTA coating demonstrated adequate EOF to carry all eight peptides past the detection window, and provided high separation efficiency and reproducibility.

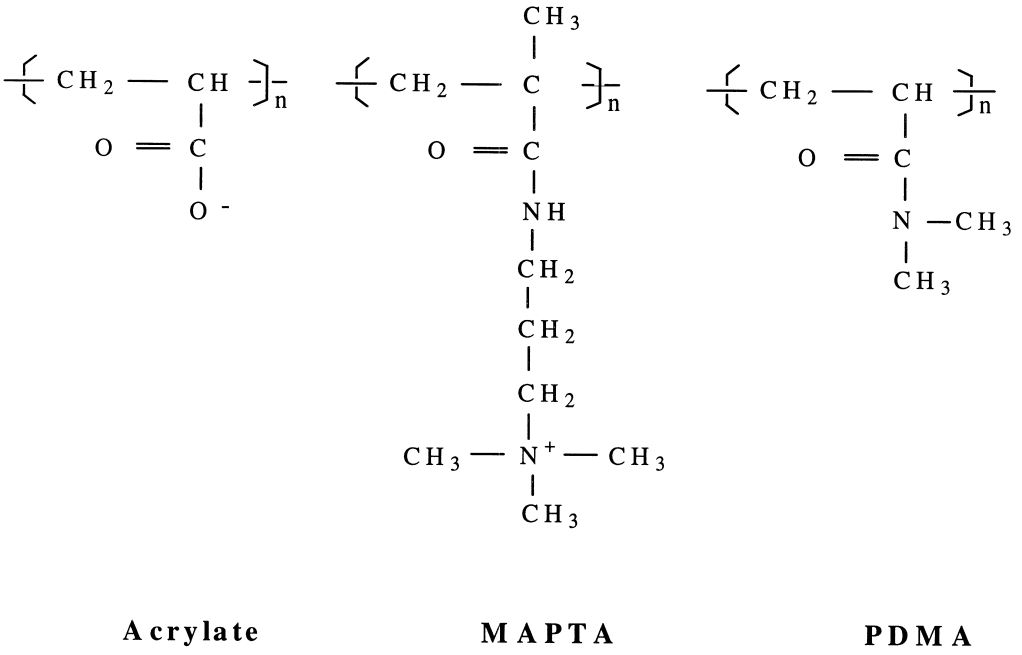

Fig. 1. Chemical structures of polymer coatings used in the current study.

2. Experimental

2.1. Chemicals and reagents

Fluorescent reagents were obtained from Molecular Probes (Eugene, OR, USA). All peptides including phosphopeptides were synthesized by the Beckman Peptide and Nucleic Acid Facility at Stanford University. The peptides were amidated on their carboxyl terminus for their eventual use inside cells to limit proteolysis by carboxypeptidases. N-terminally labeled fluorescent peptides was prepared as previously described [44]. Peptides were labeled with fluorescein by incubation with single isomer 6-carboxy fluorescein succinimydyl ester unless stated otherwise. The PKC peptide substrate F-PKC (RFARKG<u>S</u>LRQKNV) was derived from the pseudosubstrate region of PKC [45]. The CamKII peptide substrate F-CamKII (KKALHRQE<u>T</u>VDAL) was derived from the threonine 286 autophosphorylation site of CamKII [46]. The PKA peptide substrate F-PKA (KRREIL<u>S</u>RRPSYR) was derived from the cAMP response element binding protein [47]. The calcineurin substrate, F-CAL (DLDVPIPGRFDRRV<u>S</u>VAAE) was derived from the RII subunit of cAMP-dependent kinase [48]. The underlined residues are the phosphorylatable residues within the peptides. Many of the peptides adsorbed readily to glass and polypropylene, and so were stored in concentrated aliquots in siliconized polypropylene microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA, USA). Fresh dilutions were used for each run. The concentrations of the fluorescein-labeled peptides were estimated by comparison of their fluorescence (excitation 480 nm, emission 520 nm) to that of standards of hydrolyzed fluorescein succinimydyl ester. While fluorescent quenching may lead to some inaccuracy in estimating peptide concentrations by this approach, it was utilized because of the very limited quantities of the highly purified, labeled peptides used in the current study. Methacryloxy-propyltrimethoxysilane (MAPS), was obtained from United Chemical Technologies. *N,N*-Dimethylacrylamide (DMA), [3-(methacryloylamino)propyl]-trimethylammonium chloride (MAPTA), acrylic acid (HA), and 2-hydroxyethyl acrylate (HEA) were purchased from Aldrich. *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and ammonium persulfate (APS) were obtained from Bio-Rad. Fused-silica capillary tubing (50 μm I.D.× 360 μm O.D.) was obtained from Polymicro Technologies. All other reagents and materials were purchased from Fisher Scientific. ECB, a physiologic extra-cellular buffer, was composed of 135 m*M* NaCl, 5 m*M* KCl, 10 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 2 m*M* $MgCl_2$, and 2 m*M* $CaCl_2$ and adjusted to pH 7.4 with NaOH. TBE buffer was composed of 44.5 m*M* Tris base, 44.5 m*M* boric acid and 10 m*M* EDTA, pH 8.3.

2.2. Determination of relative net charges of the peptides at pH 7.4

The approximate overall charge of each peptide was estimated using the Java-based software MOLECULAR BIOLOGIST'S WORKBENCH, from the European Molecular Biology Laboratory of Heidelberg (http://members.aol.com/_ht_a/lucatoldo/myhomepage/JaMBW/). The charge from fluorescein after reaction with the peptide was estimated at −1 and this value was added to the overall charge [49]. For phosphorylated peptides, the phosphate group was estimated to add a charge of −2 to the peptide at pH 7.4 [7].

2.3. Capillary coating procedures

2.3.1. Protocol for PDMA polymer coating

PDMA-coated capillaries were prepared by a modified procedure of Hjerten and Kubo [28,29]. Pretreatment of the capillary inner surface was performed by flushing the capillary sequentially with 0.1 *M* NaOH (12 h), deionized water (4 h), 0.1 *M* HCl (12 h) and deionized water (12 h). Next the inner surface of the capillary was activated with a methacryl group by flushing at a rate of 70 μl/h with 50% methanol (2 h), then 100% methanol (2 h), followed by 5% (v/v) MAPS in methanol, and was allowed to stand overnight at room temperature. The capillary was then washed to remove unreacted MAPS with 100% methanol (2 h), 50% methanol (2 h), and then water. Polymerization was then performed on the activated inner surface of the capillaries by filling the capillary with a degassed solution of 2.5% (v/v) DMA in TBE buffer with

0.2% (v/v) TEMED and 0.5 mg/ml APS. The capillaries were flushed overnight with this solution at room temperature, and then uncoupled PDMA was removed by flushing the capillaries with water.

2.3.2. Protocol for acrylate polymer coating

Pretreatment and activation of the inner surface of the capillary with a methacryl group were performed according to the above procedure. The wash step was followed immediately by filling the capillary with a degassed solution of 3.3% (w/v) acrylate in water with 0.2% (v/v) TEMED and 0.16% (w/v) APS. For capillaries coated with acrylate copolymerized with HEA, acrylate was mixed with HEA at a 4:9 molar ratio. The capillary was then flushed overnight with this solution at room temperature. Finally uncoupled acrylate or acrylate–HEA was removed by flushing the capillaries with water.

Preparation of a pH 7, 33% acrylate stock solution: 7.2 g of acrylic acid was dissolved in 6 ml of water. A concentrated NaOH solution was added to neutralize this acidic solution to pH 7, and then brought to a total volume of 22 ml with water. A 1:10 dilution of stock was prepared on the day of use.

2.3.3. Protocol for MAPTA polymer coating

Pretreatment and activation of the inner surface of the capillary with a methacryl group were performed as above. The wash step was followed immediately by filling the capillary with a degassed solution of 2.5% (w/v) MAPTA in water with 0.2% (v/v) TEMED and 0.16% (w/v) APS. The capillary was then flushed overnight with the MAPTA solution at room temperature. Uncoupled MAPTA was removed by flushing the capillary with water.

2.4. Apparatus and separation conditions

CE and fluorescence detection was performed as described previously with the following exceptions [50]. The capillary length was 75 cm with an optical window created 50 cm from the inlet. Depending on the coating, the voltage was adjusted to achieve a current of 66 μA with ECB as the run buffer. For all runs the inlet was held at ground with the outlet at high voltage. For bare capillaries, the voltage was −12 kV. For PDMA-coated capillaries, the voltage was −6.7 kV for positively-charged peptides, and +6.7 kV for negatively-charged peptides. To achieve separation of a mixture of basic and acidic peptides in the PDMA-coated capillaries, hydrodynamic flow was used to increase the mobility of the peptides toward the detection window (see Results and discussion). The hydrodynamic flow applied to the capillary was formed by raising the fluid level surrounding the inlet 8 cm above that of the outlet. Polarity switching of the power supply (from −6.7 to +6.7 kV) during some runs was also applied (see Results and discussion). For acrylate-coated capillaries, the outlet reservoir was held at −12 kV. For MAPTA-coated capillaries, the outlet reservoir was held at +12 kV.

The samples were injected by hydrodynamic flow for 7 s with a height difference between inlet-to-outlet reservoir fluid levels of 2.5 cm. The volume loaded was calculated using Poiseulle's equation plus spontaneous fluid displacement [13,51,52]. Analytes were detected by laser-induced fluorescence as described previously [50]. Current versus voltage curves were performed for each capillary coating [13].

In experiments utilizing bare fused-silica capillaries, the capillary was prepared by sequentially washing with 0.1 *M* NaOH (12 h), water (4 h), HCl (12 h), and water (12 h). Bare capillaries were washed between electrophoretic runs (~12 column volumes) sequentially with 0.1 *M* NaOH, water, 0.1 *M* HCl, water, and ECB. The coated capillaries were washed between runs sequentially with water and ECB (~20 column volumes each). Thorough washing is required when performing CE separations of most peptides, and the reduction in number of wash steps is one advantage of coated vs. bare capillaries. All capillaries were conditioned by running CE in ECB for 30 min at the beginning of each day.

2.5. Measurement of electroosmotic flow

EOF was measured using the neutral marker, Bodipy FL propanol (0.1 *M*), as described previously [53]; μ_{eo} was calculated from the equation: $\mu_{eo} = L_d/(E \cdot t_m)$ for L_d (the capillary length to the detector), t_m (the migration time), and E (the field strength) [13].

2.6. Measurement of theoretical plates

The theoretical plates (N) were calculated from the equation: $N = 2\,(t_m \cdot h/A)^2$ with the variables defined as the peak area (A), peak height (h) and migration time (t_m) [54].

3. Results and discussion

3.1. Separation performance of peptides with bare capillaries

Separations of peptide mixtures in bare capillaries were problematic. As can be seen from Table 3, the nonphosphorylated and phosphorylated pairs of the substrate peptides F-PKC/FP-PKC and F-CamKII/FP-CamKII exhibited inconsistent migration times with RSD values between 1.5 and 2.6%. The peptides also displayed poor reproducibility with regards to peak areas (data not shown). We thus pursued the use of polymer-coated capillaries to improve separation efficiency and reproducibility for the analysis of the peptide mixtures.

3.2. Separation performance of peptides with PDMA-coated capillaries

Our initial choice for a covalently-bonded polymer was PDMA. This neutral polymer has been widely used for separation of peptides and proteins [11,26,27]. PDMA has also been found to resist fouling by a number of biological molecules, a property of importance to applications such as cellular analysis by CE where the heterogeneous contents of biological cells are loaded into the capillary [10,31]. Previous reports have described the separation of acidic or basic peptides using PDMA capillaries, but not both simultaneously [31–33,36]. This limitation is imposed because the suppressed EOF in a PDMA-coated capillary is usually inadequate to overcome the electrophoretic mobility of peptides of like charge to the outlet voltage. Thus, positively-charged peptides do not readily migrate past the detection window with an anodic outlet, and negatively-charged peptides do not with a cathodic outlet. We attempted separation of acidic and basic peptides with both anodic and cathodic outlet conditions, but the more highly-charged peptides could not be detected during single runs up to 6000 s (data not shown).

Since EOF in the PDMA-coated capillaries ($0.56 \pm 0.01 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}) was inadequate to overcome μ_{eo}, we attempted to drive all analytes to the detection window by introduction of hydrodynamic flow during the run as previously described [55–57]. Fig. 2 shows the CE separation of a mixture of acidic and basic peptides in a PDMA-coated

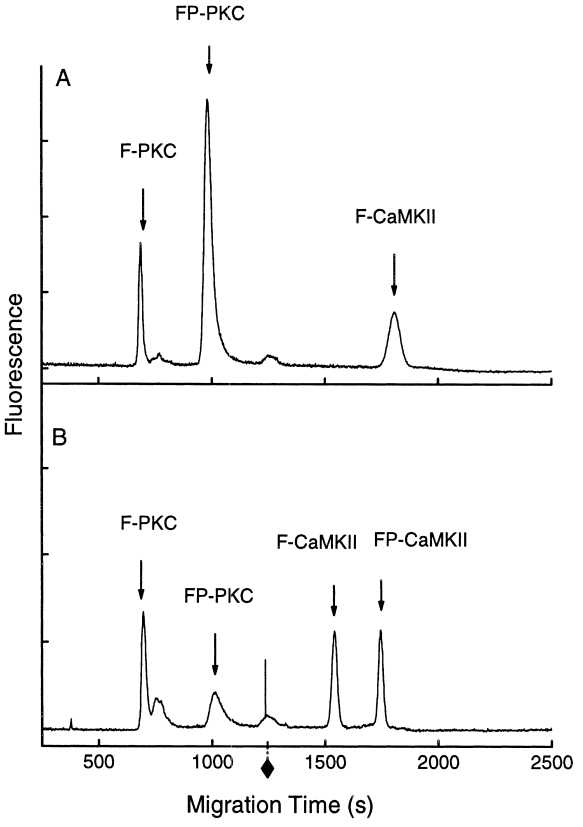

Fig. 2. Separations of acidic and basic peptides in PDMA-coated capillaries (43 cm×50 μm I.D×360 μm O.D., 25.5 cm effective length) at room temperature using ECB buffer, pH 7.4. Eight centimeters of gravity flow was applied to the capillary during electrophoresis (see text). (A) The separation of a 0.74 nl sample of F-PKC (2 nM), FP-PKC (5 nM), F-CamKII (1 nM), FP-CaMKII (5 nM), at −6.7 kV. (B) The separation of 0.74 nl of F-PKC (2 nM), FP-PKC (2.5 nM), F-CamKII (1 nM), FP-CaMKII (1 nM) using polarity switching from −6.7 kV, and then switching to +6.7 kV at 1240 s into the run (denoted by ♦) (see text).

capillary with a cathodic outlet in which gravity flow was applied to the capillary by positioning the inlet 8 cm above the outlet. As seen in Fig. 2A, two positively-charged peptides, F-PKC (+4), FP-PKC (+2) and the neutral F-CaMKII could be detected, but FP-CaMKII (−2) was not seen. In addition, the application of gravity flow reduced separation efficiency. For example, the parabolic hydrodynamic flow induced by gravity reduced the theoretical plates for F-PKC from $19.38 \cdot 10^4$ plates/m to $4.34 \cdot 10^4$ plates/m.

We then attempted a new strategy for the simultaneous separation of positively- and negatively-charged peptides—polarity switching of the outlet potential during the run. In large-volume sample stacking, polarity switching has been used as a means to increase sample loading [58,59]. In this technique, a voltage is applied to the column during stacking, and then the polarity is reversed to perform the analytical run. The resulting enhanced electrokinetic injection results in detection improvements of up to three orders of magnitude [58]. We reasoned that in our experiments the analytes loaded into the column by gravity injection would be retained by the presence of residual electroosmotic and hydrodynamic flow during the run. After detection of the positively-charged peptides, reversal of the power supply's polarity during the run could be used to drive the retained negatively-charged peptides to the detection window by μ_{eo} and continued hydrodynamic flow.

A mixture of the four peptides was introduced into the PDMA-coated capillary column. A negative outlet voltage was applied to the column to separate the positively-charged F-PKC and FP-PKC while gravity flow was applied to retain the negative peptides in the column. After detection of F-PKC and FP-PKC the run was continued for a sufficient time to drive these peptides well beyond the detection window, and then the polarity of the power supply was switched to a positive voltage of equal magnitude in order to detect the negative F-CamKII and FP-CamKII. The identities of the third and fourth peaks as the CamKII peptides were established in control experiments omitting F-PKC/FP-PKC, thus confirming that these peaks were not F-PKC and FP-PKC being driven back through the detection window by the reversal of μ_{eo} induced by the polarity switching (data not shown). Fig. 2B shows that the four peptides could be successfully separated and detected by this method with RSD values of the migration times between 0.5 and 1.3% (Table 3). Separation efficiencies were unchanged compared with those in PDMA-coated capillaries with gravity flow. While separation of the peptides was performed in shorter times, this approach still suffered from the loss of separation efficiency due to the hydrodynamic flow required to maintain all analytes in the capillary.

3.3. Separation performance of peptides with poly(acrylate)-coated capillaries

The drawbacks due to diminished EOF in PDMA-coated capillaries motivated the use of coatings that enabled production of a strong EOF. Acrylate polymers have had widespread use for industrial purposes [60], but to our knowledge based on a thorough search of the literature, we describe here for the first time the use of an acrylate polymer as a coating for CE. Capillaries coated with this negatively-charged polymer retained an EOF of equivalent magnitude to that of bare capillaries (Table 2). If desired the EOF can be tailored by copolymerization with a neutral monomer such as HEA. For example the EOF could be reduced from that provided by acrylate alone at $2.7 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} to $0.68 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}) for acrylate copolymerized with HEA (4:9) (Table 2).

Separations of acidic and basic peptides were achieved in capillaries coated with the acrylate polymer. As demonstrated in Fig. 3, a total six peptides (four positively-charged: F-PKC; PF-PKC; F-PKA; FP-PKA; one neutral: F-CaMKII; and one negatively-charged: PF-CaMKII) were easily sepa-

Table 2
Electroosmotic fluid flow

Capillary coating	μ_{eo} ($n=5$) (10^{-4} cm^2 V^{-1} s^{-1})
Bare	2.7±0.1
PDMA	0.56±0.01
Acrylate	2.7±0.06
Acrylate–HEA (4:9)	0.68±0.02
MAPTA	−1.2±0.02

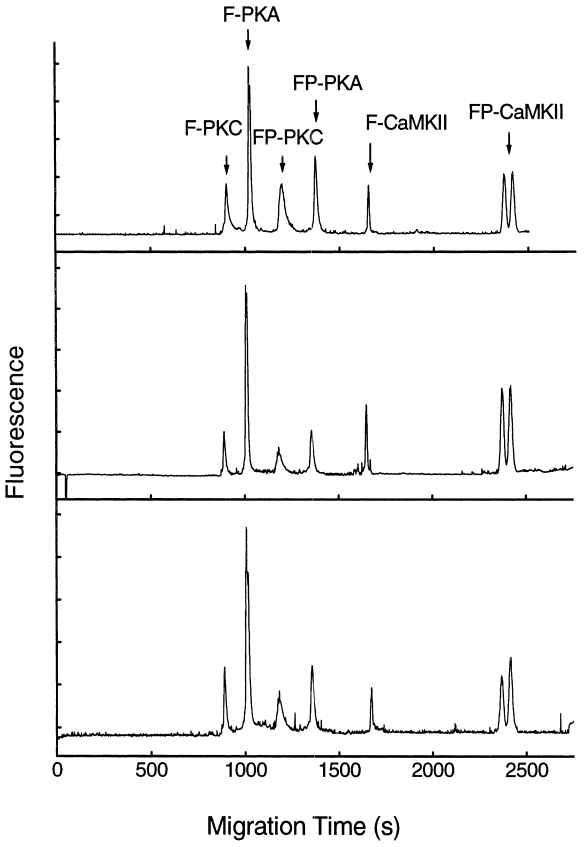

Fig. 3. Separation of six peptides in acrylate-coated capillaries [75 cm (50 cm effective length)×50 μm I.D.×360 μm O.D.] at room temperature using ECB buffer, pH 7.4, at −12 kV. For each run a 0.5-nl sample was loaded containing a mixture of acidic and basic peptides (F-PKC (0.6 n*M*), FP-PKC (2 n*M*), F-PKA (0.6 n*M*, labeled with 5′,6′mixed isomers of fluorescein), FP-PKA (2 n*M*), F-CamKII (0.3 n*M*), FP-CamKII (0.8 n*M*, labeled with 5′,6′ isomers of fluorescein).

rated in the high salt ECB buffer at pH 7.4. Additionally, separations were improved over those in bare capillaries. The RSDs for the migration times were between 0.3 and 1.2% (Table 3), and separation efficiencies were equivalent to or better than those in bare capillaries (Table 3). The higher theoretical plates particularly for the negatively-charged species and more stable migrations times suggest the adsorption of the analytes was retarded as well compared to bare capillaries. Equivalent EOF in the bare and acrylate-coated capillaries suggests that the surface charge is the same in both; therefore, it is likely that the reduced adsorption results from physical and chemical surface characteristics other than charge.

Table 3
Migration time reproducibility and separation efficiency

Peptide	RSD of migration time (%) [$n=5$]/N (10^4 plates/m)			
	Type of capillary coating			
	Bare	PDMA[a]	Acrylate	MAPTA
F-PKC	1.5/10.3	0.5/4.3	0.6/14.1	1.7/12.5
FP-PKC	2.0/13.9	1.0/1.8	0.8/2.4	0.5/5.2
F-PKA	1.5/4.7	0.4/32.1	1.4/14.4	
FP-PKA	2.3/5.5	1.0/5.9	0.6/28.4	
F-CaMKII	2.3/8.2	1.3/7.0	1.2/34.6	0.6/68.4
FP-CaMKII	2.6/8.8[b]	1.1/5.5	1.1/18.7[b]	1.5/44.3
FP-CAL	0.7/5.2	1.3/59.8		
F-CAL	0.8/4.2	1.4/38.5		

[a] 8 cm gravity flow applied to capillary (see text).
[b] For FP-CaMKII, the first peak (corresponding to one isomer of the mixed isomer label) in Figs. 2 and 4 was utilized to calculate the theoretical plate number.

3.4. Separation performance of peptides with MAPTA-coated capillaries

Despite improvements resulting from the use of the anionic acrylate coating, the most negatively charged of the peptides used in this study, FP-CAL, could not be detected under the above conditions even in runs greater than 5000 s (data not shown). We thus investigated the use of the positively-charged MAPTA polymer coating for the separation of all eight species [61]. MAPTA-coated capillaries had anodic (reversed) EOF whose magnitude was approximately half that seen in bare and acrylate-coated capillaries (Table 2). As shown in Fig. 4, all eight peptides could be separated and detected in one run. Reproducibility of electrophoresis with MAPTA-coated capillaries was good with RSDs of the migration times between 0.45 and 1.68% (see Table 3). The separation efficiencies were much improved over all other conditions used in these studies with theoretical plate numbers between 5.2 and $68.4 \cdot 10^4$ plates/m (Table 3). Surprisingly, the best two separation efficiencies obtained were for the separation of the neutral peptide F-CaMKII ($68.4 \cdot 10^4$ plates/m) and the negatively-charged peptide FP-CAL ($59.8 \cdot 10^4$ plates/m), again suggesting that peptide–wall

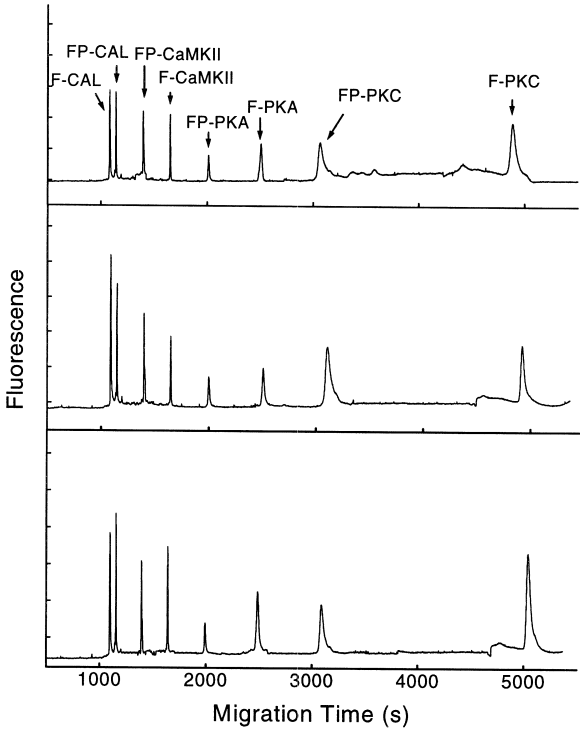

Fig. 4. Separation of eight peptides in MAPTA-coated capillaries [75 cm (50 cm effective length)×50 μm I.D.×360 μm O.D.) at room temperature using ECB buffer, pH 7.4, at 12 kV. The traces are from CE separations performed over a 3-day period in the same capillary. For each run a 0.5-nl sample was loaded containing a mixture of acidic and basic peptides (F-PKC (8 n*M*), FP-PKC (2 n*M*), F-PKA (2 n*M*), FP-PKA (1.2 n*M*), F-CamKII (0.1 n*M*), FP-CamKII (0.2 n*M*, labeled with 5′,6′ isomers of fluorescein), FP-CAL (0.1 n*M*), and F-CAL (0.1 n*M*)). Peaks 1–8 were identified to be F-CAL, FP-Cal, FP-CamKII, F-CamKII, FP-PKA, F-PKA, FP-PKC, F-PKC, respectively.

interactions might have been mediated by characteristics other than charge.

4. Conclusion

In the current study we demonstrate a variety of conditions for separating mixtures of acidic and basic peptides under the restrictions of a physiologically compatible buffer system. Approaches investigated in this study to overcome the incompatible velocities in μ_{eo} of peptides of opposite charge include: (1) employment of hydrodynamic flow; (2) application of polarity switching during electrophoresis; and (3) the use of new polymer coatings. The use of charged polymeric coatings which retain strong EOF and show reduced peptide–wall interactions appears to be the most efficient and practical means for achieving such separations. These approaches are all compatible with a living biologic cell; consequently, they should be of interest to the growing number of investigators using CE for the analysis of biologic phenomena.

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