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# Electrochromatographic Retention Studies on a Flavin-Binding RNA Aptamer Sorbent

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**Aptamers are oligonucleotides that are isolated and amplified on the basis of their recognition of a target molecule. In this study, an RNA aptamer isolated and amplified on the basis of its affinity for flavin mononucleotide (FMN) was covalently bound to the inner walls of fused-silica capillaries. This aptamer recognizes the flavin moiety of both FMN and flavin adenine dinucleotide (FAD). When an attempt was made to evaluate these capillaries according to existing theory, the theory proved to be insufficient. We describe a new method to evaluate capillaries for use in open-tubular capillary electrochromatography (OTCEC) of charged analytes, which combines OTCEC and flow-counterbalanced capillary electrophoresis. This method enabled us to extract $k'$ and evaluate $k_{CEC}$ values for these capillaries, and the dependence of these values on $Mg^{2+}$ concentration was explored. The $k'$ values for these capillaries ranged from 0.0951 to 0.2530 and from 0.0255 to 0.1118 for FMN and FAD, respectively.**

Aptamers are single-stranded oligonucleotides that are isolated and amplified on the basis of their affinity for a target molecule by "systematic evolution of ligands by exponential enrichment" (SELEX).[1,2] Aptamers have been isolated on the basis of their affinity for a diverse collection of target molecules, including small molecules,[3−5] peptide sequences,[6,7] proteins,[8,9] and other oligonucleotides.[10] The large number of possible oligonucleotide sequences and their molecular diversity make possible the isolation of aptamers that show affinity for a large variety of molecules.[11]

The use of these molecules in analyses requiring molecular recognition shows promise and has been reviewed.[12] Aptamers have found use in such techniques as flow cytometry,[13,14] sensors and biosensors,[15−18] affinity chromatography,[19−21] affinity capillary electrophoresis,[22,23] and capillary electrochromatography.[24,25] These molecules show advantages over other molecules traditionally used in such applications, including their small size and their easy and reproducible synthesis. The small size of these molecules offers two potential advantages for the use of these molecules in specific separations: an increase in achievable surface coverage and stability resulting from the simple three-dimensional structure of these molecules.

Flow-counterbalanced capillary electrophoresis (FCCE) is a technique that was originally devised to increase the efficiency and resolving power of capillary electrophoresis.[26] This technique involves using pressure in the presence of an electric field to closely control the electrokinetic migration of analytes through a capillary. In the original studies, the migration of the analytes was reversed by application of pressure in order to move the analytes back and forth across the detection window so that the analytes remained in the separation field until separation was achieved.

In the study presented here, we used a modified version of FCCE in order to adjust the net electroosmotic flow (EOF) to zero inside both aptamer-modified and bare fused-silica capillaries. An RNA aptamer consisting of 35 bases, isolated on the basis of its affinity for the flavin moiety of the small biological cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD),[27] was immobilized inside the fused-silica capillaries used

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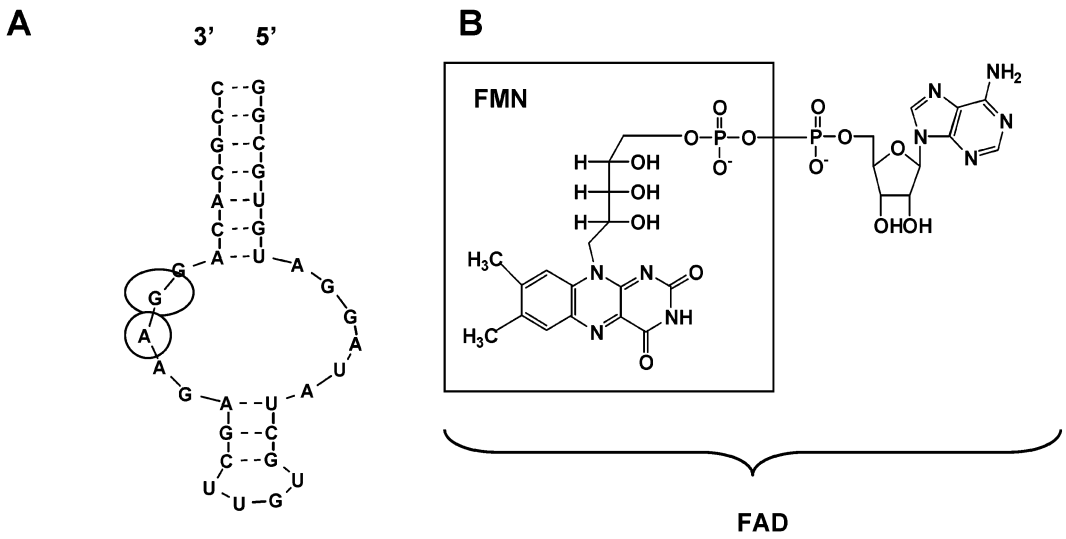


Figure 1. Sequence of the RNA aptamer (A) and its targets (B). The positions A26 and G27 are circled as referred to in the text.

for open-tubular capillary electrochromatography (OTCEC). The RNA aptamer and its targets are shown in Figure 1. Using flow-counterbalanced open tubular capillary electrochromatography (FC-OTCEC), the method described herein, the chromatographic value $k'$ for the aptamer stationary phase was experimentally determined for different mobile phase conditions.

## EXPERIMENTAL SECTION

**Chemicals and Materials.** Tris(hydroxymethyl)aminomethane (Tris), thiourea, (glycidoxypropyl)trimethoxysilane (GOPS), 1,1′-carbonyldiimidazole (CDI), phosphate buffered saline (PBS), sodium phosphate, FAD, and FMN were purchased from Sigma (St. Louis, MO). Acetone, ethyl ether, and magnesium chloride were purchased from Mallinckrodt (St. Louis, MO). The 5′-amine-modified RNA aptamer was purchased from Oligos, Etc. (Wilsonville, OR). HPLC grade acetonitrile (ACN) and xylene were purchased from Fisher Scientific (Pittsburgh, PA). The ACN was dried over 4-Å molecular sieves prior to use. All other chemicals were used as received. Fused-silica capillary tubing was purchased from Polymicro Technologies (Phoenix, AZ).

**Capillary Preparation.** The 35-base RNA aptamer was covalently bound to the inner walls of fused-silica capillary tubing using a modified version of a previously published method.[28] First, the capillaries were filled with a solution consisting of 75% xylene, 25% GOPS, and 0.1% Hünig's base by volume and placed at 80 °C overnight. Capillaries were then flushed with xylene, dried by flushing with argon, filled with dilute HCl (pH 3.5), and placed at 80 °C for 5 h. Capillaries were then flushed with several volumes each of water, acetone, ethyl ether, and argon to dry. Each was then placed at 100 °C for at least 10 hours to ensure dryness. Next, the capillaries were filled with a saturated solution of CDI in ACN and allowed to react at room temperature for 5 h. Capillaries were flushed with ACN and dried with argon before being filled with the 5′-amine-modified RNA aptamer (220 $\mu$M) in 50 mM sodium phosphate (pH 8). The capillaries were left at room temperature for 3 h, then dried with argon and stored at 4 °C. To prepare them for use, the capillaries were flushed with 0.1 M PBS for several minutes to remove any unreacted aptamer. Figure 2 shows the complete reaction resulting in the immobilization of the RNA aptamer. All capillaries used in the study were cut to 35.0 cm with a detection window burned 8.5 cm from the outlet.

**Instrumentation.** All separations were conducted on a Hewlett-Packard $^{3D}$CE equipped with a diode array detector. The capillary was kept at 20 °C during all separations. Detection was achieved online by burning the polyimide coating of the capillary to create a detection window, and data was collected at 254 nm.

**OTCEC.** Open tubular CEC studies were performed on capillaries of 50-$\mu$m i.d.. During each run, −30 kV was applied across capillaries 35.0 cm in total length. The run buffer was 20 mM Tris/HCl (pH 7.6). As different concentrations of $MgCl_2$ were added to the mobile phase, the ionic strength was kept constant at 20 mM by addition of the run buffer. Therefore, any effect the ionic strength may have on the recognition exhibited by the aptamer for its targets was factored out of the evaluation of the effect of $Mg^{2+}$ concentration.

The negative polarity applied during each run caused the electroosmotic flow to be reversed so that the analytes traveled across 8.5 cm to the detection window. Thiourea was used as the $t_0$ marker in all experiments. The polarity was reversed in these experiments as a result of the small EOF generated in aptamer-derivatized capillaries, which caused excessively long run times. Under the conditions stated, only FAD could be eluted from aptamer-derivatized capillaries. We believe this is due to both the slow EOF in these capillaries and the higher affinity the aptamer exhibits for FMN relative to FAD.

**Flow-Counterbalanced OTCEC.** Capillaries used in the FC-OTCEC experiments were also 35.0 cm in total length and 50 $\mu$m in inner diameter. In these experiments, pressure that opposed the direction of EOF was applied. During each run, −5 kV was applied while a constant pressure was applied to the inlet vial. While the direction of EOF was from the anode to the cathode, the negatively charged analytes traveled from the cathode to the anode. The contribution from EOF inside the capillary was zero as a result of the pressure counterbalance so that the velocity of

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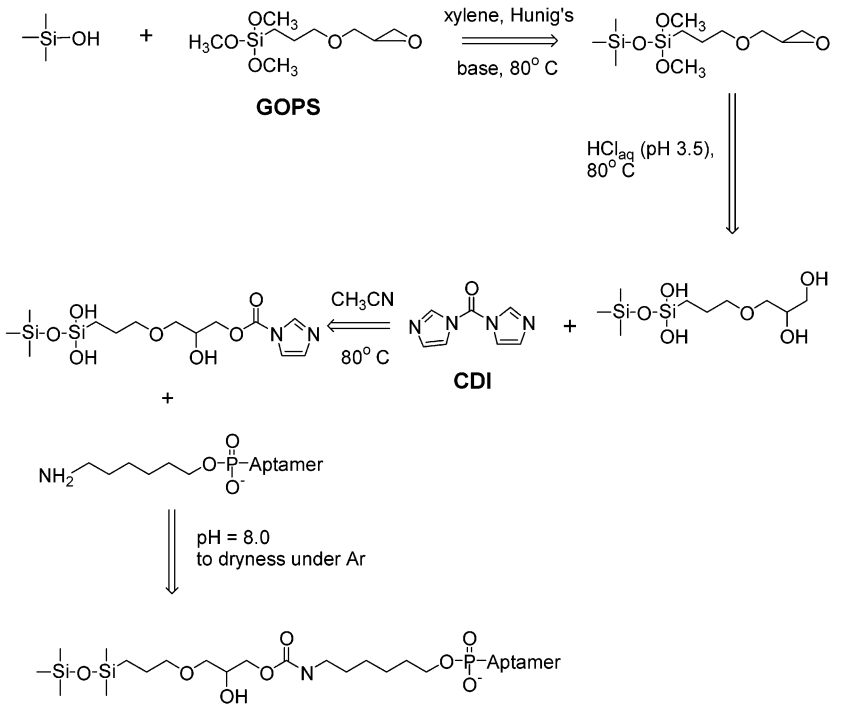


Figure 2. Scheme resulting in covalent bonding of an RNA aptamer onto the inner walls of a fused-silica capillary.

the analytes was a function of their size, charge, and extent of retention on the aptamer stationary phase.

The pressure was monitored several ways to confirm that it effectively counterbalanced the EOF. First, the EOF for the capillary was established using thiourea as the $t_0$ marker in the mobile phase under investigation while applying a potential of 5 kV. Next, the capillary was filled with the mobile phase, and each end of the capillary was placed in 5 mM Tris/HCl. A constant voltage of −5 kV was applied across the capillary while a constant pressure was applied to the inlet vial. If the pressure applied was too high or too low to counterbalance EOF, a decrease in current was observed (Figure 3). Once the necessary counterbalancing pressure was established, the $t_0$ marker was injected and flowed through the capillary using the pressure that produced a constant current in the experiments described above, but in the absence of an electric potential. This experiment was undertaken to be certain that the neutral marker eluted at the same time as it did under EOF in the absence of pressure. During runs involving only FMN or FAD, the analyte was injected at the cathode. A constant pressure was applied at the inlet while −5 kV was applied across the capillary. To make certain that the pressure was successfully offsetting EOF, the pressure was monitored to make sure that the pressure-vs-time profile was flat, meaning that there was no net flow through the capillary. FMN and FAD were injected separately into both bare fused-silica capillaries and aptamer-derivatized capillaries under a variety of mobile phase conditions. Values given for the migration times and electrophoretic mobilities of each analyte represent an average of at least 12 runs.

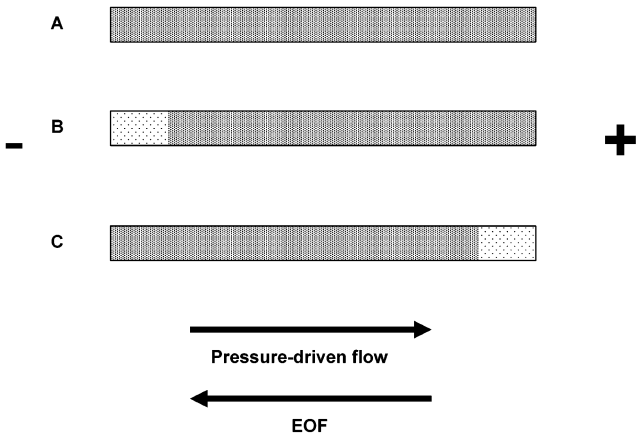


Figure 3. Schematic of the use of pressure to oppose EOF. Capillary is filled with 20 mM Tris/HCl (pH 7.6), while inlet and outlet vials are filled with 5 mM Tris/HCl (pH 7.6). (A) Pressure-driven flow is exactly equal and opposite to EOF, resulting in constant current and no net flow. (B) Pressure-driven flow is greater than EOF, resulting in the transport of electrolyte in the inlet vial into the capillary, accompanied by a decrease in current. (C) Pressure-driven flow is lesser in value than EOF, allowing electrolyte in the outlet vial to enter the capillary and current to decrease.

## RESULTS AND DISCUSSION

The 3-D structure of oligonucleotides is stabilized by the presence of divalent cations through interactions between the metal ion and $\pi$ electrons present in the oligonucleotide.[29] These interactions have been shown to affect the affinity exhibited by RNA and DNA aptamers toward their target molecules.[19,30] Therefore, when characterizing these capillaries, different concentrations of $MgCl_2$ were used to study the effect $Mg^{2+}$ might

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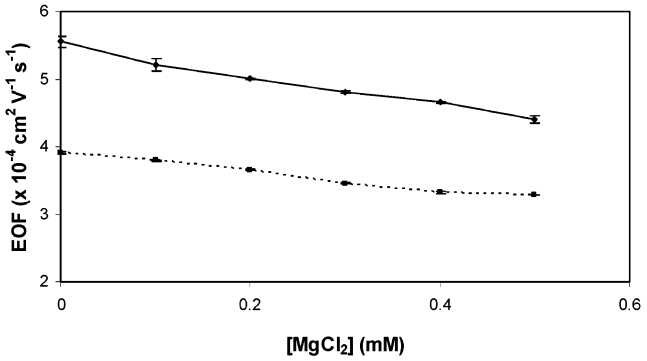


Figure 4. EOF in bare fused-silica capillaries and in aptamer-derivatized capillaries. The run buffer was adjusted to a constant ionic strength of 20 mM with the addition of Tris/HCl (pH 7.6), the neutral marker was thiourea, the capillaries were 35.0 cm in total length and 26.5 cm to detection, 5 kV was applied, hydrodynamic injection was 25 mbar for 2.5 s, and error bars represent one standard deviation for $n \geq 7$ runs. (—) Bare fused-silica capillary, (----) aptamer-derivatized capillary.

have on the affinity shown by the aptamer-derivatized capillaries for FMN and FAD.

**Open-Tubular CEC.** Open-tubular CEC experiments in which the aptamer-derivatized capillaries were compared with bare fused-silica capillaries under the same conditions were undertaken. Figure 4 shows a comparison of the electroosmotic mobility in a bare fused-silica capillary and in a capillary derivatized with the RNA aptamer. Although the aptamer is negatively charged under the separation conditions, if this charge contributes to the electroosmotic mobility generated in the capillaries, the contribution must be minimal, since the electroosmotic mobility in the aptamer-derivatized capillaries is smaller than in bare fused-silica capillaries.

The migration of charged analytes in CEC is a function of both their electrophoretic mobility ($\mu_{ep}$) and chromatographic retention. The $\mu_{ep}$ of a charged analyte is directly proportional to the applied field strength ($E$) and the charge of the analyte ($q$), and inversely proportional to the viscosity of the mobile phase and the analyte's Stokes radius. Experimentally, this value is calculated using the following equation

$$\mu_{ep} = \frac{L_T L_D}{V}\left(\frac{1}{t_A} - \frac{1}{t_0}\right) \quad (1)$$

where $L_T$ is the total length of the capillary, $L_D$ is the length to detection, $V$ is the total voltage applied across the capillary, $t_A$ is the migration time of the analyte, and $t_0$ is the migration time of a neutral marker. The extent of chromatographic retention of the analyte is characterized by the retention factor ($k'$)

$$k' = \frac{t'_r}{t_0} \quad (2)$$

where $t'_r$ is the adjusted retention time of the analyte and $t_0$ is the time necessary for an unretained analyte to travel to the point of

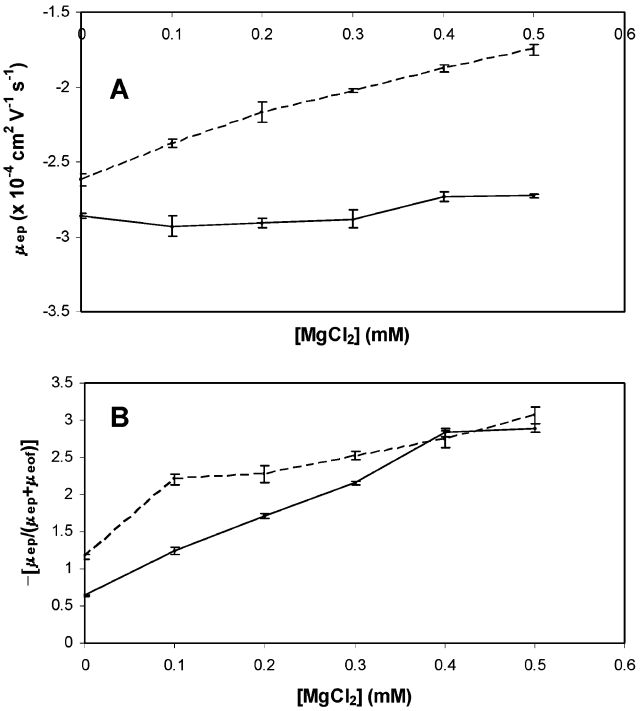


Figure 5. $\mu_{ep}$ (A) and $-[\mu_{ep}/(\mu_{ep} + \mu_{eof})]$ (B) of FAD in a bare fused-silica capillary and in an aptamer-derivatized capillary. Capillaries were 35.0 cm in total length, 8.5 cm to detection; −30.0 kV applied; run buffer kept at a constant ionic strength of 20 mM with the addition of Tris/HCl (pH 7.60); FAD concentration was 250 $\mu$M; hydrodynamic injection was 50 mbar for 1 s, and error bars represent one standard deviation for $n \geq 7$ runs. (—) Bare fused-silica capillary, (-----) aptamer-derivatized capillary.

detection. In CEC experiments, the migration of a charged analyte is given by eq 3[31,32]

$$k_{CEC} = k' - \frac{\mu_{ep}}{\mu_{ep} + \mu_{eof}}(k' + 1) \quad (3)$$

where $k_{CEC}$ is the CEC retention coefficient, $\mu_{eof}$ is the electroosmotic mobility, and the term $\mu_{ep}/(\mu_{ep} + \mu_{eof})$ corresponds to the retention of the analyte in pure CE mode. As $k'$ approaches 0, $k_{CEC}$ approaches $\mu_{ep}/(\mu_{ep} + \mu_{eof})$, and the retention mechanism is completely electrophoretic. That is, there is no chromatographic contribution to the retention of the analyte.

Using eq 3, we attempted to evaluate the extent of aptamer−target interaction inside the aptamer-derivatized capillaries. Figure 5 shows two comparisons of the two sets of capillaries. In part A, the comparison of $\mu_{ep}$ in bare fused-silica capillaries and in aptamer-derivatized capillaries is shown. It is clear that $\mu_{ep}$ of FAD in aptamer-derivatized capillaries is different from the $\mu_{ep}$ in bare fused-silica capillaries; the $\mu_{ep}$ of FAD in aptamer-modified capillaries is less negative than in fused-silica capillaries. This means that the analyte is actually moving faster inside aptamer-derivatized capillaries, which seems counterintuitive. Part B shows a comparison of the value $\mu_{ep}/(\mu_{ep} + \mu_{eof})$ in bare and modified capillaries. According to this figure, the $k'$ approaches 0 at higher

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Table 1. $k'$ Values for FMN and FAD in Aptamer-Derivatized Capillaries[a]

| [$MgCl_2$] (mM) | FMN | | | FAD | | |
|---|---|---|---|---|---|---|
| | $k'$ | SD | rel SD | $k'$ | SD | rel SD |
| 0 | 0.0951 | 0.0010 | 1.1% | 0.0255 | 0.0004 | 1.6% |
| 0.1 | 0.1979 | 0.0036 | 1.8% | 0.0534 | 0.0010 | 1.9% |
| 0.2 | 0.2530 | 0.0042 | 1.7% | 0.0535 | 0.0011 | 2.1% |
| 0.3 | 0.1877 | 0.0037 | 2.0% | 0.0645 | 0.0010 | 1.6% |
| 0.4 | 0.1533 | 0.0014 | 0.9% | 0.0688 | 0.0013 | 1.9% |
| 0.5 | 0.1753 | 0.0032 | 1.8% | 0.1118 | 0.0035 | 3.1% |

[a] Capillaries were 35.0 cm in total length and 26.5 cm to detection, 50 $\mu$m in inner diameter; −5 kV was applied while a constant pressure was applied at the inlet to counterbalance the EOF; buffer was kept at a constant ionic strength of 20 mM by the addition of Tris/HCl (pH 7.6); FAD and FMN concentration was 250 $\mu$M; hydrodynamic injection was achieved by applying 50 mbar for 1 s; snf reported standard deviation is for $n \geq 12$ data points.

$Mg^{2+}$ concentrations, since the value $\mu_{ep}/(\mu_{ep} + \mu_{eof})$ inside aptamer-modified capillaries approaches that value in bare capillaries at higher [$Mg^{2+}$]. Since it was shown that the modification of the capillaries had an effect on $\mu_{eof}$ (Figure 4) and the value $\mu_{ep}/(\mu_{ep} + \mu_{eof})$ has a dependency on $\mu_{eof}$, it is unclear whether eq 3 can be used to evaluate these capillaries in an unbiased manner.

**FC-OTCEC.** These experiments were undertaken in order to fully explore the chromatographic retention occurring on the aptamer stationary phase. In these experiments, pressure was applied across bare fused-silica capillaries and aptamer-derivatized capillaries, which effectively counterbalanced the EOF present in the capillaries. Using migration times of the analytes FMN and FAD under these conditions, $k'$ for the aptamer stationary phase was extracted in the following way,

$$k' = \frac{t_{apt} - t_{fs}}{t_{fs}} \quad (4)$$

where $t_{apt}$ is the migration time of the analyte in an aptamer-derivatized capillary and $t_{fs}$ is the migration time of the analyte in a bare fused-silica capillary. The net EOF was 0 in the experiments, so the migration of the analyte through the capillary was a function of only the size and charge of the analyte and the extent of its interaction with the aptamer. Therefore, if there was no chromatographic retention of the analyte, the migration times on bare and aptamer-modified capillaries should be identical under the same conditions. Table 1 shows the $k'$ values for FMN and FAD, which were calculated using eq 4.

The values for $k'$ show a dependence on the concentration of $Mg^{2+}$ in the run buffer. For FMN, $k'$ shows a maximum at 0.2 mM, but $k'$ for FAD increases across all concentrations. The $k_d$ values for the aptamer−FMN and −FAD complexes in solution are 0.5 and 0.7 $\mu$M, respectively.[27] The recognition exhibited by the RNA aptamer has been studied through NMR spectroscopy and molecular dynamics simulations and occurs through a base-pair interaction between an adenine in the RNA aptamer (A26 in Figure 1) and the uracil-like edge of the flavin portion of FMN.[33,34] The molecular dynamics simulation studies also reported additional H-bonding between FMN and the RNA aptamer that occurred through the movement of the phosphate group in FMN toward a guanine present in the aptamer (G27).[34] Although the molecular dynamics simulation studies took the presence of molecular water into account, the presence of $Mg^{2+}$ was not considered, whereas the NMR studies were conducted in the presence of $Mg^{2+}$. We believe that this may be the cause of the observed dependence of $k'$ on [$Mg^{2+}$] for FMN in our studies. The presence of a high concentration of $Mg^{2+}$ may shield the phosphate group of FMN so that this interaction is lessened, whereas the phosphate groups are more effectively shielded in the FAD molecule. Neither set of studies evaluated the aptamer−FAD complex.

According to eq 3 and Figure 5B, the maximum and minimum values of $k'$ for FAD should be at 0.1 and 0.4 mM [$Mg^{2+}$], respectively. However, according to Table 1, $k'$ for FAD increases with increasing [$Mg^{2+}$] through all concentrations evaluated. This result shows that the variation in electroosmotic flow for modified OTCEC capillaries makes it impossible to evaluate $k_{CEC}$ using this equation. The interaction between the aptamer stationary phase and FAD seems to affect the value $\mu_{ep}$ in OTCEC experiments (Figure 5A), and the same pattern is seen in $\mu_{ep}$ as in $k'$ (Table 1).

## CONCLUSION

Present theory has proven to be insufficient when evaluating retention of analytes in OTCEC. This is largely due to the variation of electroosmotic flow invoked by modifying the inner walls of fused-silica capillaries. We have presented an alternative method for evaluating the extent of interaction between analytes and the stationary phase in OTCEC and have extracted $k'$ values for these capillaries under conditions in which EOF has no net effect on migration. Using this method, the affinity exhibited by an immobilized RNA aptamer toward its two target molecules was characterized in different [$Mg^{2+}$] concentrations.

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