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Application of capillary electrophoresis in anion binding studies: Complexation and separation of nitrate and nitrite by an azacryptand

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Abstract

Capillary electrophoresis (CE) was employed for studying the complexation of an azacryptand with nitrate and nitrite in aqueous solution. CE separation of a mixture of nitrate and nitrite with 10 mM acetate buffer (pH 3.3) showed two peaks at the retention times of 2.8 and 3.1 min for nitrate and nitrite, respectively. However, when the ligand (2 mM) was added to the running buffer, the peaks emerged in the reverse order and at shorter retention times of 2.7 and 2.5 min for nitrate and nitrite, respectively. The longer retention time for nitrate compared with nitrite indicates a stronger complex formation between the ligand and nitrate, that reduces the migration speed of nitrate as compared with the less strongly bound nitrite. The ¹H NMR titrations of **L** with these two anions at the pH 3.3, gave the binding constants (log *K*), 3.75 and 4.23, for nitrite and nitrate, respectively which were in consistence with the results obtained from the CE method.

Keywords

Anion binding; capillary electrophoresis; azacryptand; nitrate and nitrite separation; molecular recognition

Introduction

Selective anion binding is an emerging area of research because of the many important roles that anions play in environmental, biochemical, and health related issues.[1-2] Among the different classes of anion receptors, polyazamacrocycles have been shown to effectively bind inorganic anions including halides and oxoanions, as well as biologically important anions such as dicarboxylates and nucleotides.[2] The analytical methods employed to study the binding properties of ligands for anions include pH-potentiometry, NMR, UV-VIS, fluorescence titration studies in solution, as well as X-ray crystallography in the solid state. [3-10] However, to our knowledge, capillary electrophoresis (CE) has not been applied to investigate the anion binding properties of synthetic ligands. Because of the widespread application of azacryptands in anion binding, we chose a readily obtainable thiophene-based

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SUPPORTING INFORMATION ¹H NMR and ¹³C NMR of **L**, stacks of ¹H NMR titrations, Job plots and experimental procedure for capillary preconditioning.

cryptand **L** to study its efficiency for separating nitrate and nitrite from their mixture by the aid of CE as shown in Fig. 1. Herein, we report the complexation effect of **L** on the migration and separation of nitrate and nitrite in CE, and ^1H NMR binding studies performed at pH 3.3.

Nitrate and nitrite are widely distributed in vegetables, meat products, biological fluids, and natural aquatic environments. Nitrite salts of sodium and potassium are used as primary ingredients in curing meat. However, the salts are toxic when used in excessive amounts and may lead to the formation of potential carcinogens.[11] In blood, nitrate and nitrite are the end products of the final pathway of nitric oxide (NO) metabolism in vertebrates.[12] Nitric oxide, produced by specific enzymes, plays an important role in various physiological processes related to immunity and neurotransmission.[13] Because these two anions have significant impacts on biological functions, identification and analysis of these anions become crucial. Several methods, such as ion chromatography (IC),[14] high performance liquid chromatography (HPLC),[15] and flow injection analysis (FIA)[16] were developed for the determination and analysis of nitrate and nitrite. As a high-speed separation method, capillary electrophoresis (CE) has been increasingly utilized to analyze anionic substances. Nitrite and nitrate have similar electrophoretic mobilities, therefore, numerous methods based on capillary ion analysis (CIA) are used in order to separate them.[17-21] For example, an electroosmotic flow modifier[17] containing running buffer or specialized coated capillary[18] can ensure net direction of anions migrating towards the anode at the detection side and enhance ion separation. Other methods include conductivity detection which involves the use of more sensitive detection of ions to lower LOD (lower limit of detection),[19] transient isotachopheresis–capillary electrophoresis (tITP–CE) method that increases the limits of direct UV absorption detection,[20] and microchip CE method.[21] In this study, we applied a simple CE method to investigate the complexation effect of an anion receptor **L** on the migration and separation of nitrate and nitrite in solution.

Experimental

Synthesis

L. The ligand **L** was prepared following the literature method.[22] 1.00 g (6.84 mmol) of *tris*(2-aminoethyl)amine and 1.45 g (10.3 mmol) of 2,5-thiophenedicarboxaldehyde were dissolved separately in 200 mL of CH_3OH . Both solutions were added simultaneously to a 400 mL of CH_3OH at 0°C over 3 h. After stirring the solution for another day, the solvent was evaporated under reduced pressure. The residue was dissolved in 100 mL of CH_3OH , and 1.80 g (47.6 mmol) of NaBH_4 was added to the solution. Stirring was continued for 24 h at room temperature. Solvent was removed under reduced pressure to yield a yellowish

product that was dissolved in 100 mL of water, and was extracted by CH_2Cl_2 (3×50 mL). The combined organic layers were collected, dried by MgSO_4 and concentrated to give a yellowish oil. The product was purified by column chromatography (neutral alumina, 2% CH_3OH in CH_2Cl_2 , $R_f = 0.90$). Yield: 0.85 g, 40%. FAB-MS: m/z 617 $[\text{HL}]^+$. ^1H NMR (300 MHz, CDCl_3 , TMS): δ 6.82 (s, 6H, ArH), 3.92 (s, 12H, ArCH₂), 2.74 (t, $J(\text{H,H}) = 4.9$ Hz, 12H, NCH₂CH₂), 2.62 (t, $J(\text{H,H}) = 5$ Hz, 12H, NCH₂CH₂). ^{13}C NMR (75 MHz, CDCl_3 , TMS): δ 141.8 (C_{Ar}), 125.9 (HC_{ar}), 53.7 (ArCH₂), 48.1 (NCH₂CH₂), 47.0 (NCH₂CH₂). Anal. Calcd. for ($\text{C}_{30}\text{H}_{45}\text{N}_8\text{S}_3$): C, 58.40; H, 7.84; N, 18.16. Found: C, 58.53; H, 7.97; N, 18.61.

$[\text{H}_6\text{L}]^{6+} \cdot 6\text{TsO}^-$. Hexaprotonated ligand was prepared from the reaction of 62 mg (0.10 mmol) **L** with 175 mg (0.92 mmol) *p*-toluenesulfonic acid monohydrate in 2 mL of methanol. The white powder was immediately formed, that was collected by filtration and was washed with diethyl ether. Yield: 95 mg, 66 %. ^1H NMR (300 MHz, D_2O , TSP): δ 7.68 (d, $J = 8.4$ Hz, 12H, TsCH₂), 7.35 (d, $J = 8.5$ Hz, 12H, TsCH₂), 7.16 (s, 6H, ArH), 4.37 (s, 12H, ArCH₂), 3.11 (t, $J = 6.4$ Hz, 12H, NCH₂CH₂), 2.75 (t, $J = 6.4$ Hz, 12H, NCH₂CH₂), 2.37 (s, 18H, TsCH₃). Anal. Calcd. for ($\text{C}_{72}\text{H}_{90}\text{N}_8\text{S}_9\text{O}_{18}$): C, 52.60; H, 5.52; N, 6.82. Found: C, 52.90; H, 5.45; N, 6.55.

Capillary electrophoresis (CE) studies

Chemicals—All solutions, electrolytes and standards were prepared using 18.2 Ω -cm Milli-Q water (Milli-Q Plus Water System, Simpak, Millipore). An acetate buffer solution with pH 3.3 was diluted to the final concentrations of 10 mM and 20 mM respectively, and used as a running buffer. Water was filtered by Millipore system. Prior to CE analysis, all anionic samples and running buffers were filtered through a Fisherbrand Nylon 0.20 μm /25 mm syringe filter. Standard solutions of 10 mM NaNO_2 and KNO_3 were prepared by dissolving in 10 mM/20 mM acetate buffers, and the solutions were sequentially diluted to obtain a calibration curve. The concentration of the ligand buffer solution was adjusted to 1 mM, 2 mM and 4 mM with the 10 mM/20 mM acetate buffers.

Instrumentation—The analysis was carried out on a Hewlett-Packard^{3D} Capillary Electrophoresis System with a diode array UV detector and HP ChemStation software. A 75 μm I.D.×360 μm O.D. fused-silica capillary was used for separation, in which a total length was 65 cm and effective length was 57 cm to the detection window. A match flame was used to burn a small section of coating to form a window for optical detection 8 cm from the end of the capillary. UV detection was achieved at 200 nm (BW 16 nm), 214 nm (BW 20 nm), 254 nm (BW 30 nm) and 284 nm (BW 40 nm). Samples were injected into the capillary by pressure mode (3 s, 15 mbar, home value is 4 mm). Separations were carried out by using a negative power supply with the applied voltage set at 25.0 kV, current controlled at 100 μA and temperature controlled at 25°C. The data and electropherograms were recorded and processed by ChemStation software.

NMR titration studies

The binding properties of **L** for nitrate and nitrite were evaluated by ^1H NMR titrations with a 300 MHz NMR instrument (Bruker) in D_2O at pH =3.3, using the corresponding sodium salts. Initial concentrations of the ligand and anions were 2 mM, and 20 mM, respectively, and the pH of both solutions was adjusted with TsOH and NaOH, and calculated from the observed pD of the solution in D_2O using the correlation, $\text{pH} = \text{pD} - 0.4$.²³ Sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3,-*d*₄ acid (TSP) dissolved in D_2O , was used as an external reference in a sealed capillary tube. Each titration was performed by 12 measurements varying the $[\text{anion}]_0/[\text{ligand}]_0 = 0 - 10$ at 25°C. The association constant *K* was calculated

by fitting of the change in aliphatic proton signals with a 1:1 association model[24]. Error limit in K was less than 15%.

Results and Discussion

Synthesis

The synthesis was carried out following the published method used for other bicyclic analogues[25] and involved high dilution cyclization of tren and 2,5-thiophenedicarboxaldehyde at 0°, yielding a cyclic Schiff base (imine). The imine was reduced using NaBH₄ and the resulting crude product was purified by column chromatography on neutral alumina, to yield the desired ligand. The calculated yield (40%) is comparable to its bicyclic analogues.[25] Reaction of **L** with TsOH gave hexaprotonated ligand that was confirmed by the relative integration of ¹H NMR spectra.

Electrophoresis Studies

Optimization of CE separation conditions—In order to optimize the separation condition for nitrate and nitrite by electrophoresis method, the effects of buffer and concentration were investigated in solution varying pH and concentrations of both ligand and buffer using nitrate and nitrite standards. Based on the experimental results, the best separation was achieved using 2 mM ligand and 10 mM acetate buffer at pH 3.3.

Separation of nitrate and nitrite with or without the ligand in the running buffer—Separation of nitrite and nitrate was performed by capillary electrophoresis using 2 mM ligand and 10 mM acetate buffer at pH 3.3. Fig. 2A shows the electropherogram for pure nitrate appearing in a single peak at the retention time of 2.8 min, when the acetate buffer was used. Switching from nitrate to nitrite under the same conditions, a peak appeared at the retention time of 3.1 min that was slower by 0.3 min than that observed for nitrate. Using an equimolar amount of nitrate and nitrite solution, two distinct peaks were seen at the retention time of 2.8 and 3.1 min, that corresponded to nitrate and nitrite, respectively (Fig. 2B). The influence of **L** on the migration and separation of the two anions, was investigated using the ligand coated on the background electrolyte (BGE) of the capillary. The results as shown in Fig. 3, indicate that the retention time for the migration of nitrate was 2.7 min, while that for nitrite was 2.5 min. In this case the shorter retention time was observed for nitrite rather than nitrate, demonstrative of a reversal of migration order from that observed in the pure acetate buffer. The same order and interval of retention time was also reflected for the equimolar mixture of two anions with 2.7 and 2.5 min for nitrate and nitrite, respectively.

These observations clearly demonstrate that **L** affects both migration order and retention time of the anions. In the first case, where pure acetate buffer (pH=3.3) was present, nitrate was observed to migrate faster than nitrite. This could be due to the different acidity of nitrous and nitric acids. Because nitrous acid is a weaker acid than nitric acid, it is partially ionized in water, resulting in the lower apparent charge density to nitrite and smaller *charge to size* ratio. Consequently, electrophoretic mobility of nitrite is expected to be lower than that of nitrate.[26] Similar results were found by Melanson *et al.* showing the lower mobility of nitrite compared to nitrate at low pH buffers in CE.[27] However, when ligand buffer was applied on BGE, the electrophoretic mobilities of nitrate and nitrite increased and the migration order was reversed. There are two factors that should be taken into consideration. Firstly, when the negative polarity is applied at inlet end, the electroosmotic flow (EOF) moves toward the *inlet end*, resulting in a decrease of electrophoretic mobility of analyte anions. Secondly, since the ligand is a positively charged species, its presence on BGE decreases the effective electrophoretic and EOF mobilities simultaneously. The two factors

with the opposite vector offset each other to an extent and influence the overall migration speed. Under the experimental condition, **L** enhanced the electrophoretic mobilities of both anions, a result that can be attributed to the reduced EOF. Again, EOF is associated with another factor termed *zeta* potential, that is developed at the interface of the ligand and BGE in the capillary. Multiple positive charges on the ligand at low pH greatly increase ionic strength of BGE and compress the double layer. Thus the *zeta* potential is expected to be decreased, resulting in the weakening of EOF. The reverse of migration order can be accounted for the strong complexation of **L** for nitrate, that results in the reduction of electrophoretic mobility of nitrate as compared with the less tightly bound nitrite. The complexation effect was not adequate to offset the weak EOF but strong enough to reverse the order of appearance for nitrate and nitrite.

NMR titrations

In order to rationalize the results obtained in CE, we proceeded to examine the complexation ability of **L** for nitrate and nitrite by ^1H NMR titration studies using its tosylated salt in D_2O at pH 3.3. The gradual addition of an anion solution resulted in a significant downfield shift of the aliphatic protons with the highest $\Delta\delta = 0.13$ and 0.09 ppm for nitrate and nitrite, respectively. The NMR titrations provided the best fit for a 1:1 binding model (Fig. 4), yielding binding constants ($\log K$), 3.75 and 4.23 for nitrite and nitrate, respectively. The significant difference in chemical shifts as well as the observed curve fitting model (1:1) indicates the formation of an inclusion complex^{2b,7b,9b} instead of simple ion pair complexes.²⁸ This assumption is also supported by our recent report for the chloride complex of **L** forming an inclusion complex in both solution and solid phases.²² The 1:1 complexation was further confirmed by the Job plots of the ligand showing a maximum at a 0.5 mole fraction for both anions in D_2O at the same pH (see supporting information). The binding constant of **L** for nitrate is higher by a factor of four-fold than that for nitrite under similar conditions and is considerably higher than that found in *m*-xylyl ($\log K$ 3.63) and furan (Log K 3.74) analogues for nitrate binding measured by ^1H NMR titrations at pH 3.0. [7b]

The NMR titrations were performed to justify the results that were obtained in CE studies at pH 3.3, showing slower electrophoretic mobility of nitrate due to the strong complexation. As seen in the literature, the nitrite anion has been analyzed by CE method at the pH ranging from 3.0 to 3.5.²⁹ Since the NMR studies were done in D_2O , therefore the pH was adjusted to 3.3 that was calculated from the observed pD using the equation, $\text{pH} = \text{pD} - 0.4$.²³ As discussed earlier, HNO_2 is a weaker acid than HNO_3 and thus only partially dissociated at pH 3.3. Therefore, the nitrite interacts more weakly with the ligand, as expected. In CE studies the acetate buffer was used to maintain the pH, while in the NMR solution tosylates served as counter anions, and also the solvents were different. Therefore a direct comparison can not be made between the two methods. However, the observed binding data are fairly in agreement with CE studies where the relative mobility of nitrate became slower due to the strong complexation with the immobilized ligand.

CONCLUSIONS

We have applied a facile capillary electrophoresis (CE) method to study anion binding to an octaazacryptand **L** that was added on the CE running buffer. The pH of the running buffer was set low (at 3.3) so that the cryptand be protonated that was a precondition of **L** for hosting an anion. Our results clearly showed that nitrate mobility is hampered as compared to nitrite. The decreased mobility is the result of strong complex formation between **L** and nitrate. In order to verify this assumption, NMR titration studies were performed to measure binding affinity of the ligand at the same pH, as it was in CE studies. The calculated binding data from NMR measurements that gave the higher binding constant for nitrate with nitrate/

nitrite selectivity around 4, also supports the results obtained from CE. The CE is well documented as a fast and cost effective technique for separation of anions, however we demonstrated in this work that the migration time and the elution order of anions could be controlled using an anion receptor in CE. While there is a growing interest in anion binding hosts in different areas of chemistry and biology, researchers working in this field are taking advantage of different types of analytical techniques to understand the principle underlying host guest chemistry. Our results from the capillary electrophoresis studies on anion binding would be an indication for future applications of this technique in the field of anion coordination chemistry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental diagram of CE for the separation of nitrite and nitrate using **L** coated on the background electrolyte (BGE).

Figure 2. Electropherograms for nitrate (**A**) and a mixture of nitrate and nitrite (**B**) solutions using 10 mM acetate buffer at pH 3.3. Anion concentrations were 1.0 mM.

Figure 3. Electropherogram for nitrate (A) and a mixture of nitrate and nitrite (B) solutions using 2 mM ligand buffer on a background electrolyte (BGE) at pH 3.3. Anion concentrations were 1.0 mM.

Figure 4.

^1H NMR titration curves of **L** with NO_3^- (●) and NO_2^- (■) in D_2O at pH 3.3. The changes in resonances are shown for the chemical shifts of aliphatic H1 (= NHCH_2) and H2 (NCH_2).