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Aptamer-Modified Monolithic Capillary Chromatography for Protein Separation and Detection

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A capillary chromatography technique was developed for the separation and detection of proteins, taking advantage of the specific affinity of aptamers and the porous property of the monolith. A biotinylated DNA aptamer targeting cytochrome c was successfully immobilized on a streptavidin-modified polymer monolithic capillary column. The aptamer, having a G-quartet structure, could bind to both cytochrome c and thrombin, enabling the separation of these proteins from each other and from the unretained proteins. Elution of strongly bound proteins was achieved by increasing the ionic strength of the mobile phase. The following proteins were tested using the aptamer affinity monolithic columns: human immunoglobulin G (IgG), hemoglobin, transferrin, human serum albumin, cytochrome c, and thrombin. Determination of cytochrome c and thrombin spiked into dilute serum samples showed no interference from the serum matrix. The benefit of porous properties of the affinity monolithic column was demonstrated by selective capture and preconcentration of thrombin at low ionic strength and subsequent rapid elution at high ionic strength. The combination of the polymer monolithic column and the aptamer affinities makes the aptamer-modified monolithic columns useful for protein detection and separation.

Aptamers are synthetic nucleic acids that specifically bind to the targets.[1–3] Since their discovery in the 1990s, aptamers have shown diverse applications to biosensor development, molecular interaction studies, and potential development of therapeutic drugs.[3–7] They have also been used in capillary electrophoresis (CE), capillary electrochromatography (CEC), and affinity chromatography for separation, detection, and purification of targets.[8] Like DNA affinity chromatography for DNA-binding proteins,[9,10] aptamer affinity chromatography has potential applications for a variety of targets using aptamers that can be generated by systematic evolution of ligands by exponential enrichment (SELEX).[1–3] Several research groups have demonstrated the use of aptamer-based chromatography for the separation of small molecules,[8] such as adenosine and its analogues,[11,12] amino acids and their derivatives,[13–17] polypeptides,[18] and flavin mononucleotides.[19,20] Recent work has shown the separation of macrobiomolecues using aptamer affinity chromatography.[21–27] These separations were performed on either packed columns[11,12,15–18,21,26,27] or open tubular capillaries.[13,14,19,20,22–25] A limitation of the open tubular capillaries is the small loading capacity because of the limited surface area of the inner wall. Packed columns, on the other hand, either have large void volumes when packed with large particles or have high back pressure when packed with small particles.

In contrast, monolithic columns consist of a single piece of rigid porous structure prepared by in situ polymerization or consolidation inside the column.[28,29] Monolithic columns offer

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several distinct advantages, including large mass transfer rate, low back pressure, easier preparation than the packed columns, and larger loading capacity than the open tubular capillaries. As an alternative to conventional packed columns, monolithic columns have attracted considerable attention for use in chromatography.[30–34]

The objective of this research is to develop an affinity aptamer monolithic capillary chromatography technique for protein separation and detection, taking advantage of both monolithic columns and aptamers. A DNA aptamer targeting cytochrome c was chosen as a model to demonstrate the feasibility. Proteins were separated based on their different binding affinities to the immobilized aptamers. This approach can be applied to a diverse range of targets that have aptamers of varying affinity.

EXPERIMENTAL SECTION

Preparation of Capillary Monolithic Columns. Polymer monolithic columns were prepared according to the published method.[35] First, a fused silica capillary (100 μm i.d., 360 μm o.d., Polymicro Technologies, Phoenix, AZ) was silanized by γ-methacryloxypropyl trimethoxysilane (γ-MAPS, Sigma) in order to anchor the polymer monolith on the capillary wall.[35] Glycidyl methacrylate (GMA, Aldrich) and trimethylolpropane trimethacrylate (TRIM, Aldrich) were chosen as monomers because TRIM could enhance the mechanical stability of the monolith.[35] The polymerization reaction solution was prepared by mixing 150 μL of GMA, 50 μL of TRIM, 510 μL of cyclohexanol, 90 μL of dodecanol, and 2 mg of azobisisobutyronitrile (AIBN, Sigma). The mixture was ultrasonicated for 20 min and degassed with nitrogen for 10 min. Subsequently, the silanized capillary (40 cm) was filled with the polymerization mixture to a total length of 8.5 cm. Both ends of the capillary were sealed with rubber septa (Supelco), and the capillary was placed in a 50 °C water bath to allow for a polymerization reaction for 12 h. After the reaction was completed, the monolithic column was connected to a HPLC pump, and the column was flushed with methanol to remove the porogenic solvents and the unreacted reagents. Monolithic columns were characterized by scanning electron microscopy (SEM, Hitachi S-2500 scanning electron microscope, Tokyo, Japan). The SEM images of the monolith inside capillary (Figure S1 in the Supporting Information) showed similar porous structure, pore size, and cluster size to those reported previously.[35]

The polymer monolith was further characterized by measuring the pore size distribution and porosity. The monolith was prepared under the same polymerization conditions as described above except that a larger amount of monolith was prepared in a stainless steel column (100 mm long × 4.6 mm i.d., obtained from Phenomenex Inc.). The monolithic rod formed inside the column was then pushed out and dried at 70 °C for 24 h. The porosisty of the monolith was determined by mercury intrusion porosimetry (Autopore IV 9500 mercury porosimeter, Micromeritics Inc.). The distribution profile of the pore size (Figure S2 in Supporting Information) showed a maximum centered at about 550 nm, which is consistent with the reported results for the same preparation method. [35]

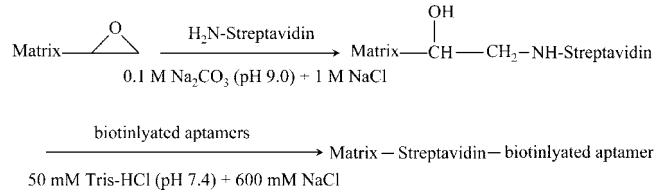

Figure 1. Reaction scheme for immobilization of aptamers on the polymer monolithic column.

Proteins. Cytochrome c (from horse heart), human serum albumin (HSA), hemoglobin (Hb, from bovine blood), apotransferrin, and human immunoglobulin G (IgG) were obtained from Sigma. Human alpha thrombin was purchased from Haematologic Technologies Inc. (Essex Junction, VT). Human serum (product number, P2918) was obtained from Sigma. Rat liver tissue lysate (nondenatured; product number, L038W2) was purchased from Abnova (Taipei City, Taiwan).

DNA Aptamers. The 61-mer DNA aptamer targeting cytochrome c (5′-AGT GTG AAA TAT CTA AAC TAA ATG TGG AGG GTG GGA CGG GAA GAA GTT TAT TTT TCA CAC T-3′)[36] was synthesized by Integrated DNA Technologies Inc. (Coralville, IA). A biotin label was attached to the 5′ end of the aptamer, and the biotinylated aptamer was purified by HPLC. Prior to immobilization of the aptamers, the biotinylated aptamer solution (14 μM aptamers, dissolved in 50 mM Tris-HCl + 20 mM KCl + 600 mM NaCl, pH 7.4) was heated at 90 °C for 3 min followed by cooling at room temperature. The heat treatment was a common practice applied to allow the aptamers to form the desired conformation through renaturation.

Immobilization of DNA Aptamers. To immobilize aptamers on monolithic columns, we first attached streptavidin on the monolithic column and then used the streptavidin to capture the biotinylated aptamers (Figure 1). Briefly, the monolithic column was connected to a syringe pump and flushed with water for 0.5 h at 80 μL/h and then flushed with 40 μL of sodium carbonate buffer solution (0.1 M, pH 9.0). Next, 200 μL of streptavidin (Cortex Biochem, San Leandro, CA) solution (1 mg/mL streptavidin, 0.1 M Na_2CO_3 + 1 M NaCl, pH 9.0) was pumped through the monolithic column with a syringe pump for 24 h at room temperature. The column was then rinsed with sodium carbonate buffer solution (0.1 M, pH 9.0) to remove the unreacted streptavidin and flushed with Tris-HCl buffer (50 mM Tris-HCl + 20 mM KCl + 120 mM NaCl, pH 7.4). Finally, 50 μL of biotinylated aptamer solution was pumped through the streptavidin-modified monolithic column for 2.5 h. The column was ready to use after it was rinsed with Tris-HCl buffer solution (50 mM Tris-HCl + 20 mM KCl + 120 mM NaCl, pH 7.4) to remove any unbound aptamers. An advantage of using the biotinylated aptamer and the streptavidin on the monolithic column is that the same column could be immobilized with different aptamers, simply by adding or changing different biotinylated aptamers.

Chromatography. The pressure-driven chromatographic separation was performed on a HP^{3D}CE (Agilent, Waldbronn, Ger-

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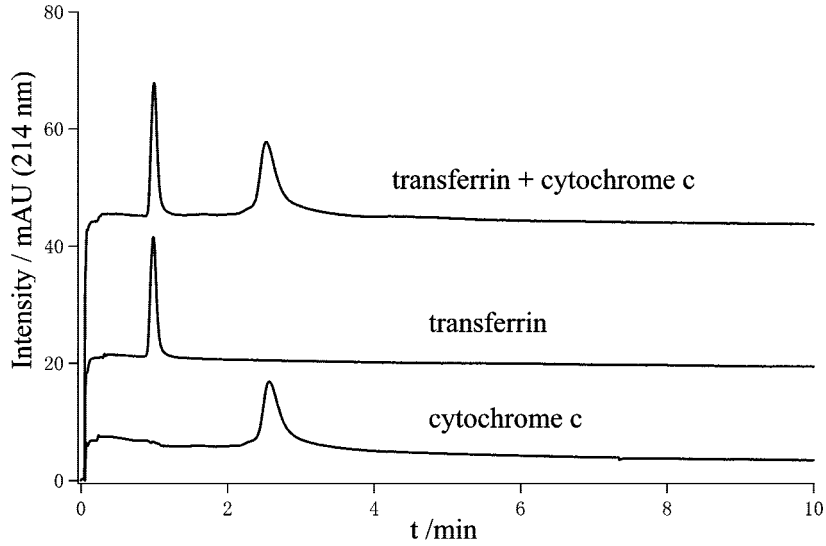

Figure 2. Chromatograms of cytochrome c and transferrin on an aptamer-modified monolithic column. Samples containing cytochrome c (0.25 mg/mL) and transferrin (0.25 mg/mL) were injected at a pressure of 8 bar for 5 s. The separation was carried out at 20 °C, using a mobile phase containing 50 mM Tris-HCl, 20 mM KCl, and 120 mM NaCl (pH 7.4). Absorbance was measured at 214 nm.

many). A short piece from the hollow end of the 40 cm capillary was cut off, and the remaining capillary with a total length of 32.5 cm was placed in the capillary cassette of the HP^{3D}CE. From the injection end, 8.5 cm was filled with the monolith and the rest of the capillary was hollow. A detection window was made at the end of the monolith portion of the capillary by carefully removing the outer polymer coating of the capillary with a razor blade. Samples were injected by applying a pressure of 8 bar at the outlet (as defined in the HP^{3D}CE instrument) for 5 or 10 s. Protein separations were achieved by applying a pressure of 8 bar on the outlet and using the aptamer binding buffer solution (50 mM Tris-HCl + 20 mM KCl + 120 mM NaCl, pH 7.4) as a mobile phase. Absorbance at 214 nm and at 407 nm (for cytochrome c) were monitored. The column temperature was kept at 20 °C.

RESULTS AND DISCUSSION

Separation of Cytochrome c on the Aptamer-Modified Monolithic Column. A 61-mer DNA aptamer targeting cytochrome c was used as a model to demonstrate the concept of protein separation on an aptamer-modified monolithic column. This aptamer can bind to cytochrome c with a dissociation constant (K_d) around 4.6 μM.[36] We initially tested the chromatography retention behavior of cytochrome c, as well as several abundant, nonspecific proteins, including human serum albumin (HSA), human immunoglobulin G (IgG), transferrin, and hemoglobin (Hb). While cytochrome c was retained on the column (2.4 min), transferrin eluted at the void time (1.0 min) (Figure 2). Similarly, Hb, HSA, and IgG were not retained on the column and were eluted at the void time. As a control, we prepared a monolithic column with streptavidin but without any immobilized aptamers. All the tested proteins including cytochrome c eluted off at the void time (data not shown). These results indicate that the columns modified with the aptamer for cytochrome c can preferentially retain cytochrome c.

In order to estimate the K_d of immobilized aptamers and the coverage density of the aptamers on the capillary column, we conducted frontal analysis. (Details on the frontal analysis are shown in Supporting Information.). Transferrin was eluted out at the void time (V_0), while cytochrome c had a longer retention on the column. As expected, with the increase of the concentration of cytochrome c ($[A]_0$) from 0.12 to 1.0 mg/mL, the volume (V) required to saturate the column decreased. Thus, the position front of cytochrome c shifted to shorter times with the increase of the concentration of cytochrome c (Results are shown in Supporting Information, Figure S3.). A plot of $1/[A]_0(V - V_0)$ versus $1/[A]_0$ was linear (Figure S3B, Supporting Information) from which the total number of aptamers immobilized on the column (B_t) was obtained to be ~110 pmol. The coverage density of aptamers on the monolithic column was determined to be ~164 pmol/μL (The 8.5 cm long monolith inside the capillary of 100 μm i.d. has a geometric volume of 0.67 μL.). As a comparison, the amount of immobilized aptamers in an open tubular capillary column (100 μm i.d., 47 cm) was ~43 pmol[25] and the coverage density was 12 pmol/μL. Thus, the immobilization capacity on our monolithic column is about 14 times higher than that on the open tubular capillary. This increase is significant considering that the length of our monolithic column (8.5 cm) was only $^1/_5$ of the open capillary column (47 cm). The coverage density of aptamers on our monolithic column is comparable to that obtained on the capillary column packed with microbeads, which was about 204 pmol/μL.[11]

The measured K_d value of cytochrome c using the above frontal analysis method was determined to be ~150 μM, which is about 30 times the reported value.[36] The higher observed K_d was initially thought to be because of different buffers used in the two studies. However, when the optimum binding buffer for the aptamer selection (containing 1% DMSO and 0.03% Triton-100, pH 8.0) was applied as the mobile phase, there was no significant change in the retention time of cytochrome c and no indication of improved K_d. The exact reason for the difference in the observed K_d and the previously reported K_d values is not clear. It is possible that the immobilization of aptamer on the column could lower the apparent binding affinity of the aptamer, although Kennedy's group did not observe a decrease in binding affinity of the aptamer

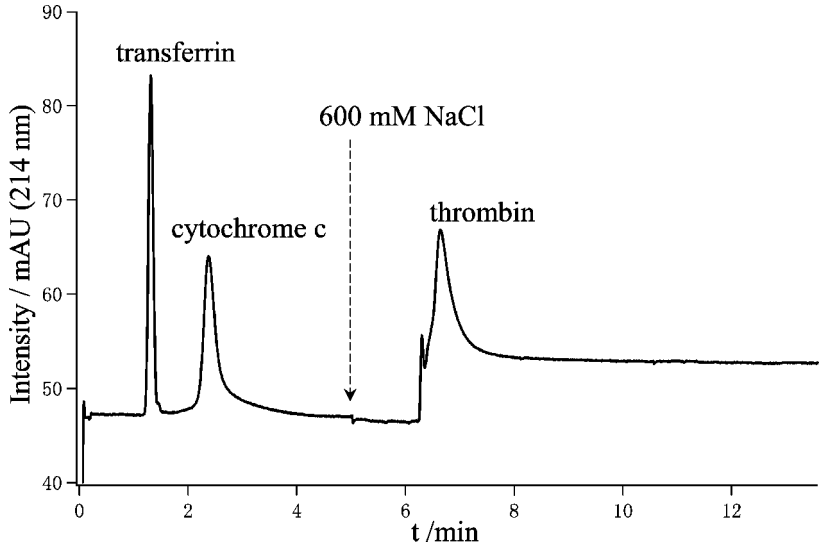

Figure 3. A chromatogram from the analysis of a mixture containing 0.33 mg/mL transferrin, 0.33 mg/mL cytochrome c, and 0.33 mg/mL thrombin. A step gradient elution program involved the first 5 min running with a mobile phase containing 50 mM Tris-HCl, 20 mM KCl, and 120 mM NaCl (pH 7.4) followed by an increase of NaCl concentration to 600 mM in the second mobile phase for the remainder of the separation.

after its immobilization on chromatographic beads.[11] Recently entrapment strategies have shown the benefit of retaining the binding affinity of the entrapped molecules.[37] Entrapment of aptamers in the sol–gel (silica monolith) may provide an alternative approach to the immobilization of aptamers in order to maintain the binding affinity.[37]

We also tested cytochrome c with various concentrations on the aptamer-modified monolithic column. In the range of 0.06 to 1 mg/mL, the peak areas are proportional to the concentration of cytochrome c (Results are shown in Supporting Information, Figure S4.). The concentration detection limit, based on the peak signal to baseline noise ratio of 3, was estimated to be 0.01 mg/mL (1 μM) cytochrome c. This corresponds to a mass detection limit of 40 fmol for an injection volume of 40 nL (5 s).

Retention of Thrombin on the Aptamer-Modified Monolithic Column. The aptamer targeting cytochrome c has a G-quartet structure, so it is possible that this aptamer can also bind to thrombin because the aptamers selected for thrombin contained a G-quartet motif.[38,39] Therefore, we tested the behavior of thrombin on the monolithic column modified with the aptamer for cytochrome c. We found that thrombin was indeed retained on the column and that thrombin could not be eluted out with the mobile phase for cytochrome c (50 mM Tris-HCl + 20 mM KCl + 120 mM NaCl). However, by increasing the concentration of NaCl in the mobile phase to 600 mM, thrombin could be successfully eluted out. Figure 3 shows the separation of a mixture containing cytochrome c, transferrin, and thrombin. After the elution of transferrin (1 min) and cytochrome c (2.4 min) for 5 min, the concentration of NaCl in the mobile phase was increased from 120 to 600 mM and thrombin was eluted out at approximately 2 min after the change of the mobile phase. It shows that the aptamer affinity column can successfully separate three proteins. The dissociation of thrombin from the column can be attributed to the reduction of charge interactions between aptamers and thrombin under the high concentration of salts. The K_d of thrombin was estimated to be 10 μM from the frontal analysis (data not shown). To confirm the capture of thrombin, the elution fractions were collected and analyzed by MALDI-MS (data not shown). A comparison of the MALDI-MS spectra from the analytes of the elution fractions and the standard thrombin sample confirmed the identity of thrombin eluted from the affinity column.

To detect the thrombin that was captured on the aptamer affinity column, we used a step-gradient elution by increasing the concentration of NaCl to 600 mM (Results are shown in Supporting Information, Figure S5.). In the range of 1.7 (0.06 mg/mL) to 109 μM (4 mg/mL), the peak areas are linear with the concentrations of thrombin (See Supporting Information, Figure S5B). The detection limit of thrombin based on the peak signal to the baseline noise ratio of 3 was estimated to be 0.5 μM or 40 fmol for an injection volume of 80 nL. To further improve the detection limit, we took advantage of the porous properties of the affinity monolithic column and carried out selective capture and preconcentration of thrombin. By introducing larger volumes (20 min injection instead of 10 s injection) of sample solutions containing lower concentrations of thrombin (14–270 nM), we were able to preconcentrate the thrombin. A detection limit of 4 nM was achieved. This represents an improvement of 120-fold due to the preconcentration.

Detection of Proteins in Complex Sample Matrix. To determine if the aptamer-modified monolithic columns can be used for the analysis of proteins in a complex sample matrix, cytochrome c and thrombin were spiked into a human serum sample or a rat liver tissue lysate sample diluted with mobile phase (50 mM Tris-HCl + 20 mM KCl + 120 mM NaCl, pH 7.4) 10 times. Figure 4 shows good separation and recovery for the cytochrome c in the diluted human serum sample. A large peak appearing at the void time resulted from the abundant proteins in the serum

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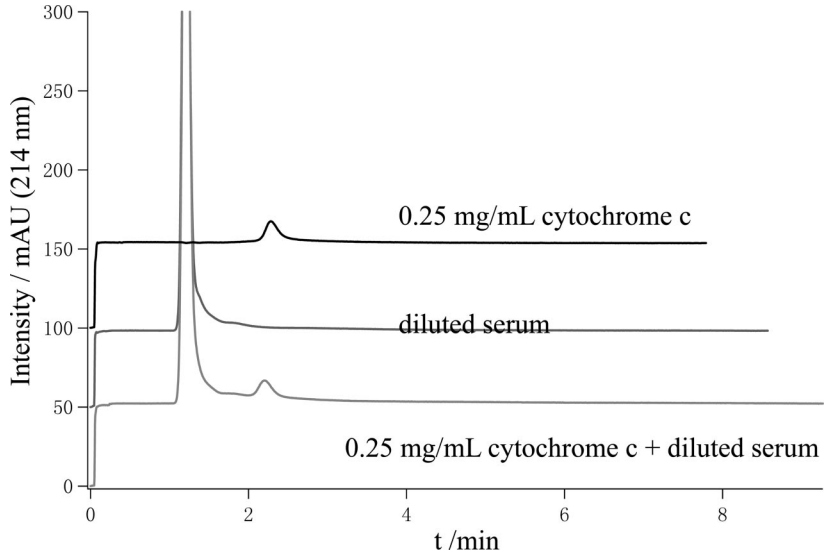

Figure 4. Detection of cytochrome c spiked in a diluted human serum sample. The same conditions as in Figure 2 were used.

sample, such as serum albumin, IgG, and transferrin. In the serum sample containing added cytochrome c, a peak was observed matching the retention time of cytochrome c, and the recovery was quantitative. The tailing peak of the unretained proteins in the serum sample had some effect on the detection of cytochrome c when the detection wavelength of 214 nm was applied. However, when 407 nm was used as a detection wavelength, at which cytochrome c has a specific and strong absorbance band resulting from the heme group,[40] the possible interference from the serum sample was reduced greatly. With an injection time of 5 s, the concentration detection limit of cytochrome c in serum was estimated to be 2 μM. The normal level of cytochrome c in human serum was reported to be <25 ng/mL (~2 nM),[41,42] below the detection limit of the present method using UV absorption detection.

We also attempted to determine cytochrome c in liver tissue lysate and found that its concentration was below the detection limit. Cytochrome c spiked into 10-fold diluted liver tissue lysate was successfully detected, and no interference was found (Data are not shown.).

The detection of thrombin spiked in the human serum sample diluted 10 times with mobile phase (50 mM Tris-HCl + 20 mM KCl + 120 mM NaCl, pH 7.4) was also achieved following the loading and elution steps. (Results are shown in Supporting Information, Figure S6.). The dilute serum sample matrix did not have any effects on the thrombin detection. Using the preconcentration function of the monolithic column, we were able to detect thrombin in dilute serum spiked with 140 (2 min injection) and 30 nM (20 min injection) thrombin. However, the normal levels of thrombin in human blood have been reported to be on the order of nanograms per milliliter (1 ng/mL corresponds to ~27 pM),[43] below the detection limit of the present method with UV absorption detection. Further improvement in the detection limit, for example, by using laser induced fluorescence detection, is needed to achieve quantitative measurements of thrombin in human blood.

The monolithic columns modified with aptamers showed good stability for repeated testing over more than 2 months. The affinity monolithic column was used for several hours every week, and the performance of the column did not change over 8 weeks. Continued flushing with the binding buffer solution for 5 h did not affect the quality of the monolithic column. The frontal analysis experiments for cytochrome c and thrombin and the injection of diluted serum sample did not influence the performance of the monolithic column. When multiple monolithic columns from the same batch were used, analyses of cytochrome c (or thrombin) on three columns showed a relative standard deviation of 6% from the retention time measurements. When the column was not in use, it was stored at 4 °C, filled with binding buffer solution, and sealed on both ends. The column did not have detectable changes in the separation of proteins after storage for more than 2 months, indicating its stability and suitability for routine applications.

In conclusion, we have developed aptamer-modified monolithic capillary columns to separate proteins based on their different binding affinities to the aptamers. Cytochrome c and thrombin were well separated from the unretained proteins, such as transferrin, serum albumin, and IgG by using the polymer monolithic capillary column modified with DNA aptamer targeting cytochrome c. Determination of cytochrome c and thrombin in diluted serum samples showed no interference from the sample matrix. The technique could potentially be used to separate other compounds by immobilizing a single aptamer or a mixture of aptamers targeting various compounds. Another potential application of the monolithic affinity aptamer columns is their use for preconcentration of target compounds to improve sensitivity and to remove sample matrix interference.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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