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Differentiation and Detection of PDGF Isomers and Their Receptors by Tunable Aptamer Capillary Electrophoresis

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Tunable aptamer capillary electrophoresis (CE) techniques were developed to enable the separation and detection of platelet derived growth factor (PDGF) isomers and their receptors. Using an aptamer that formed a stable complex with the B chain but not with the A chain of PDGF, we were able to tweak the electrophoretic mobilities of the PDGF isomers for their separation. PDGF-AB bound to a single aptamer molecule was well resolved from PDGF-BB bound to two aptamer molecules. Simultaneous determination of 50 pM of two isomers was accomplished in a single analysis. Furthermore, PDGF-AB was used as a connector to bring receptor α and fluorescent aptamer into a single complex molecule. As a result, the formation of a (receptor α)-(PDGF-AB)-(fluorescent aptamer) ternary complex enabled the detection of the receptor α by tunable aptamer CE. A competitive assay was developed to determine receptor *β*, making use of the competition between the receptor *β* and fluorescent aptamer in binding to PDGF-BB. Detection limits were 0.5 nM for PDGF receptor α and 3 nM for receptor *β*. Determination of PDGF isomers and their receptors in diluted serum samples showed no interference from the sample matrix.

Platelet derived growth factor (PDGF) is one of the critical growth factors that regulate cell growth and division.[1−3] PGDF is composed of two disulfide-linked polypeptide chains designated A and B.[4] The native protein occurs as the homodimers AA and BB or the heterodimer AB. The dimeric isomers PDGF-AA, AB, and BB are differentially expressed in various cells, and their biological functions are mediated through binding to two cell surface proteins, PDGF receptors α and *β*.[5−7] Differences exist in isoform binding to each receptor. The receptor α binds to all three PDGF isomers, whereas the receptor *β* binds only to the PDGF-AB and PDGF-BB isomers with high affinity.[8−10] It is essential to determine the composition of PDGF isomers and their receptors in order to understand their functions and their interactions. A variety of aptamer-based methods have been demonstrated for the determination of PDGF-AB and BB.[11−17] However, all these methods allowed the determination of only the total amount of PDGF-AB and BB. They are not able to differentiate and detect PDGF-AB and BB separately in a mixture.

We have recently developed a tunable aptamer capillary electrophoresis (CE) technique for multiple protein analysis.[18] The key concept is tuning the electrophoretic mobility of proteins with DNA aptamers to achieve efficient separation of multiple proteins. Aptamers are highly negatively charged, and each nucleotide carries nearly −1 charge under the pH conditions typically used for capillary electrophoresis separation.[19] This unique property makes aptamers appropriate to serve as effective charge modulators, modifying the electrophoretic mobility of specific proteins. In free-zone capillary electrophoresis, the electrophoretic mobility of a molecule is proportional to its mass-to-charge ratio. Aptamers of different sizes (composed of different number of nucleotides) have a similar mass-to-charge ratio, thus migrating through the capillary at a similar mobility. In contrast, the size of a protein is much larger than that of a nucleotide (∼300 Da, −1 charge). Binding of a protein to an aptamer leads to complexes having a

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substantially different mass-to-charge ratio than those of the unbound protein and aptamer. Thus, affinity CE techniques based on aptamer binding to proteins have proven to be highly successful.[20−25] Furthermore, aptamers could be easily synthesized to have desired lengths. As a result, the electrophoretic mobility of specific proteins could be rationally controlled by binding to aptamers of varying nucleotide length. Using four aptamers to tune the electrophoretic mobilities of four specific proteins, we have successfully separated and detected four proteins in a single capillary electrophoresis analysis.[18]

The objective of the present work was to further develop tunable aptamer capillary electrophoresis techniques enabling the separation and detection of PDGF isomers and their receptors. The strategy was to tune electrophoretic mobility of PDGF isomers by binding them with one or two aptamer molecules. We took advantage of the discovery that aptamers for PDGF-AB and BB bind only to PDGF-B chain with high affinity.[26] By using aptamers as charge modulators to modify the electrophoretic mobility, we were able to separate the two PDGF-aptamer complexes: PDGF-AB isomer bound to a single aptamer molecule and PDGF-BB isomer bound to two aptamer molecules. To detect PDGF receptor α, we made use of its interaction with PDGF-A chain and measured the ternary complex (receptor α)-(PDGF-AB)-(fluorescent aptamer). Furthermore, the competition of receptor β with aptamer for binding to PDGF-B chain rendered the development of a competitive assay for receptor β.

EXPERIMENTAL SECTION

Reagents. Recombinant human PDGF-AA, PDGF-AB, PDGF-BB, PDGF receptor α, and PDGF receptor β were all obtained from R&D Systems (Minneapolis, MN) in lyophilized form, free from carrier proteins. Bovine serum albumin (BSA) and human serum were purchased from Sigma (Oakville, ON). PDGF isomers were reconstituted in 4 mM HCl containing 0.1% BSA to prepare a stock solution of 1 μM. The PDGF receptor stock solution at 5 μM was prepared by reconstitution of receptors in 1×PBS buffer containing 0.1% BSA. The stock solutions were stored at −20 °C when not in use. PDGF binding aptamers, 20t-4 (5′-AGGGCGCGTTCTTCGTGGTTACTTTTAGTCCCG-3′) and 36t-4 (5′-CAGGCTACGGCACGTAGAGCATCACCATGATCCTG-3′), and a nonspecific 49 mer oligonucleotide (5′-TGGTCTTGTGTGGCTGTGGCTATGTCTGATCTTAATCCACGAAGTCACC-3′) were synthesized and purified by Integrated DNA Technologies (Coralville, IA). The 6′-FAM label was attached directly to the 5′ end of aptamers, and fluorescently labeled aptamers were purified by reversed-phase HPLC. The 10×TG was obtained from Bio-Rad Laboratories (Mississauga, ON). 1×TG buffer (25 mM Tris and 192 mM glycine, pH 8.3) was diluted with deionized water from 10×TG and then adjusted to desired pH by 1 M NaOH. All other reagents were commercially available analytical grade.

Capillary Electrophoresis. A laboratory-built capillary electrophoresis laser induced fluorescence (CE-LIF) system was used in this work.[27−29] The separation was conducted by using 40 cm long uncoated fused silica capillaries (20-μm i.d., 150-μm o.d. Polymicro Technologies, Phoenix, AZ) at room temperature. Samples were electrokinetically injected into the capillary at a voltage of 18 kV for 5 s, and a running voltage of 18 kV (450 V/cm) was employed to drive separation. The running buffer was 1×TG (pH 8.5) unless otherwise stated. To remove any possible adsorption of proteins from the inner surface of the capillary and to maintain reproducible CE separation, after every 5 consecutive sample injections, the capillaries were treated by running 0.02 M NaOH for 5 min, followed by water for 3 min and running buffer for 5 min, all running at an electric field of 300−450 V/cm. All CE data were analyzed using Igor Pro software (version 4.04, WaveMetrics, Lake Oswego, OR).

Under the optimized conditions, five replicate analyses of (PDGF-AB)-(aptamer 20t-4) complex, (PDGF-BB)-(aptamer 20t-4) complex, and (receptor α)-(PDGF-AB)-(aptamer 36t-4) complex give the following migration times (mean ± SD): 3.45 ± 0.06 min, 3.66 ± 0.05 min, and 2.88 ± 0.07 min, respectively. Relative standard deviations from 3−5 replicate determinations of PDGF isomers and receptors were lower than 8.0%.

Incubation of Aptamers and Proteins. To place aptamers in their desired conformation, the 5 μM aptamer stock solution in 10 mM Tris·HCl (pH 7.4) + 1 mM $MgCl_2$ was treated at 80 °C for 5 min followed by cooling slowly to room temperature before use. The sample incubation buffer was 10 mM Tris·HCl (pH 7.4) containing 1 mM $MgCl_2$ and 0.2% BSA. To form complexes, the appropriate volumes of aptamer and protein stock solutions were mixed and diluted with incubation buffer to obtain the desired concentrations of aptamers and proteins. The final sample solution was 50 μL. Before injection into CE, the sample solution was incubated at 37 °C for 20 min.

Serum Sample Analysis. Frozen serum samples were thawed in a water bath at 30 °C and then kept on ice. Prior to analysis, 0.5 mL of the serum sample was centrifuged at 10,000 rpm for 10 min to remove any precipitate. The appropriate volumes of aptamer, nonspecific DNA, and protein stock solutions were mixed with 5 μL of serum, and the incubation buffer was then added to produce a final volume of 50 μL. Samples were incubated and analyzed as indicated above.

RESULTS

Determination of PDGF Isomers by Tunable Aptamer Capillary Electrophoresis. PDGF isomers (AA, AB, and BB) are difficult to separate from one another by free-zone CE because of their similar electrophoretic mobility. To enable their CE separation and LIF detection, we introduced fluorescently labeled aptamers to bind with the PDGF proteins. Formation of the complexes makes the proteins amenable for detection by LIF (Supporting InformationFigure S1).

Two aptamers, 20t-4 and 36t-4, are known to bind to the PDGF B chain ($K_d \sim 10^{-10}$ M) with a much higher affinity than to the

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A chain ($K_d > 10^{-8}$ M).[26] Therefore, PDGF-AB and BB isomers form stable complexes with fluorescently labeled aptamers 20t-4 and 36t-4, and these complexes can be detected using CE-LIF (Supporting Information Figure S1). Furthermore, because PDGF-AB has one B chain to bind with a single aptamer molecule and PDGF-BB has two B chains to bind with two aptamer molecules, we are able to tune the mobility of the two complexes so that PDGF-AB and BB isomers can be separately detected.

We have compared the use of aptamers 20t-4 and 36t-4 as fluorescent probes for tunable CE-LIF analyses of PDGF-AB and PDGF-BB isomers. Our results show that the use of aptamer 36t-4 produced smaller complex peaks for the analyses of both PDGF-AB (Supporting Information Figure S1B) and PDGF-BB (Figure S1C). A possible reason is because aptamers 20t-4 and 36t-4 may bind to the different sites on the surface of the PDGF-B chain. The PDGF-B chain is responsible for the basic properties (pI 9.5−10) of both PDGF-AB and BB. If the surface positive charges of the PDGF-B chain were not covered efficiently by the binding to the aptamer 36t-4, the complex could be adsorbed on the capillary surface. Therefore, the use of 36t-4 resulted in smaller complex peaks for analysis of PDGF-AB and BB (Supporting Information Figure S1), although 20t-4 and 36t-4 have similar K_d to the PDGF-B chain. Consequently, 20t-4 was chosen for separation of PDGF-AB and BB.

Supporting Information Figure S2 shows that the PDGF-AB bound to a single aptamer 20t-4 is well resolved from the PDGF-BB bound to two aptamer molecules. PDGF-BB has a smaller molecular weight (25 kDa) than PDGF-AB (27 kDa) and they have a similar pI value (9.5−10.0). Under the pH of the running buffer (pH 8.5), the unbound PDGF-BB is expected to have a faster mobility than PDGF-AB. However, because aptamers carry highly negative charges (each nucleotide carries a −1 charge), their binding to proteins can modulate the electrophoretic mobility of the proteins. Upon binding to two aptamer molecules, the PDGF-BB aptamer complex carries more negative charges than the PDGF-AB-aptamer complex, which results in the slower migration of PDGF-BB (3.56 min) through the capillary than PDGF-AB (3.42 min). Therefore, through binding to one or two aptamer molecules to achieve tunable separation, PDGF isomers AB and BB can be detected in a single CE/LIF analysis.

To optimize the conditions for the detection of PDGF-isomers, we tested the effect of running buffer pH (Supporting Information Figure S3) and Mg^{2+} concentration (Supporting Information Figure S4), because both are considered important for the separation and formation of PDGF-aptamer complexes. We found that 1 mM Mg^{2+} in the incubation solution and a running buffer pH 8.5 were appropriate. Using the optimized conditions for incubation, separation, and detection, we examined the dynamic range of analyses for both PDGF isomers AB and BB. Figure 1 shows a series of electropherograms from the analysis of PDGF isomers in mixture solutions containing varying concentrations of PDGF isomers (0.5−50 nM) and 300 nM of aptamer 20t-4. Calibration curves, constructed using peak areas as a function of the protein concentration, were linear for the determination of both PDGF-AB ($r^2 = 0.998$) and PDGF-BB ($r^2 = 0.994$). A linear dynamic range of 2 orders of magnitude (0.5−50 nM) was obtained for both isomers, and this dynamic range could be extended to higher concentrations of proteins by using proportionally higher concentrations of the aptamer.

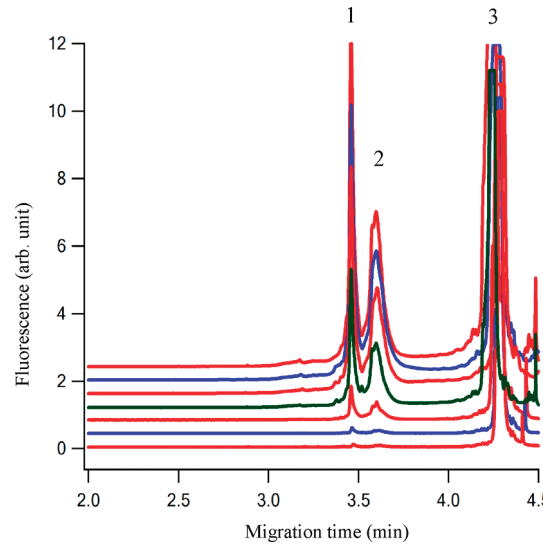

Figure 1. Electropherograms showing the determination of PDGF-AB and BB isomers. The concentrations of PDGF-AB and PDGF-BB isomers were 0.5, 2, 5, 10, 30, 40, and 50 nM, respectively, from the bottom to the top traces. Peak 1 is the complex of PDGF-AB with fluorescent aptamer 20t-4; peak 2 is the complex of PDGF-BB with two fluorescent aptamer 20t-4 molecules; and peak 3 is due to the unbound fluorescent aptamer.

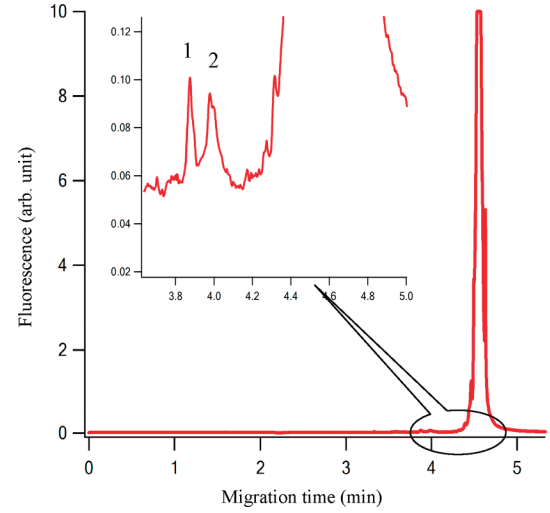

Figure 2. An electropherogram showing the detection of 50 pM of PDGF-AB and PDGF-BB isomers. Peak 1 is the complex of PDGF-AB with fluorescent aptamer 20t-4; peak 2 is the complex of PDGF-BB with two fluorescent aptamer 20t-4 molecules; and peak 3 is due to the unbound fluorescent aptamer. The concentrations were 50 pM for both PDGF-AB and PDGF-BB and 100 nM fluorescently labeled aptamer 20t-4.

The binding of fluorescent aptamers to nonfluorescent proteins makes proteins amenable for highly sensitive laser-induced fluorescence detection. Detection limits, defined as the concentration equivalent to three times the standard deviation of noise, were 50 pM for both PDGF-AB and PDGF-BB, as shown in Figure 2.

The applicability of the assay to sample analysis was demonstrated by the determination of spiked PDGF isomers in a 10-fold diluted human serum sample. The human serum was diluted to decrease the ionic strength of sample and the potential

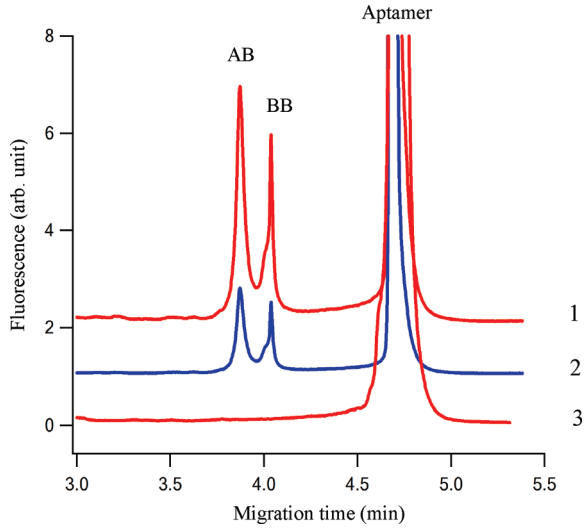

Figure 3. Electropherograms showing determination of PDGF-AB and PDGF-BB isomers in the diluted serum. The concentrations of PDGF-AB and PDGF-BB were (1) 25 nM, (2) 10 nM, and (3) 0 nM. The concentration of fluorescently labeled aptamer 20t-4 was 250 nM.

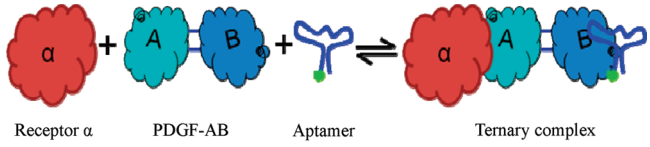

Figure 4. Schematic showing the formation of a (receptor α)-(PDGF-AB)-(aptamer) ternary complex for the determination of receptor α.

interference from the abundant proteins in the serum. A nonspecific and nonfluorescent 49-mer oligonucleotide was added into the sample to reduce any nonspecific binding of the serum proteins to the fluorescent aptamer. No apparent interference was observed when spiked isomers were detected in a 10-fold diluted human serum sample, as shown in Figure 3. Recoveries ranged from 96% to 115%.

Determination of PDGF Receptor α. PDGF receptor α can bind to PDGF-A chain.[26] This information combined with our results showing that aptamers 20t-4 and 36t-4 form detectable complexes with only the PDGF-B chain (Supporting Information Figure S1) suggests that it is possible to develop an affinity assay for the receptor α. Specifically, we made use of the PDGF-AB isomer as a connector, bringing the receptor α and the fluorescent aptamer into a single complex molecule. The formation of a (receptor α)-(PDGF-AB)-(aptamer) ternary complex enables the laser-induced fluorescence detection of receptor α (Figure 4).

We first compared the use of aptamers 20t-4 and 36t-4 to form (receptor α)-(PDGF-AB)-(fluorescent aptamer) complexes for the determination of receptor α. We found that aptamer 36t-4 was preferred because a higher sensitivity was achieved with this aptamer (Supporting Information Figure S5). We also compared three incubation modes on the formation of the (receptor α)-(PDGF-AB)-(aptamer) complex. In the first experiment, the receptor α was first incubated with PDGF-AB for 10 min, followed by the addition of aptamer 36t-4 and another 10 min incubation. In the second experiment, the PDGF-AB was incubated with aptamer 36t-4 for 10 min, and then receptor α was added for the next 10 min incubation. In the last experiment, the three components were mixed at the same time and incubated for 20 min. The results indicated that all three conditions produced little difference in the formation of the (receptor α)-(PDGF-AB)-(aptamer) complex. Thus, two binding events, the binding of receptor α to PDGF-AB and the binding of aptamer 36t-4 to PDGF-AB, are quite compatible and do not affect each other.

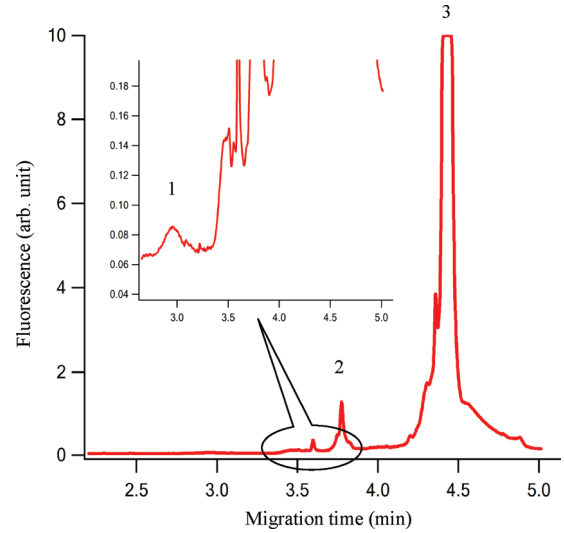

Figure 5. An electropherogram showing the detection of 0.5 nM PDGF receptor α. The concentrations of PDGF-AB and fluorescent aptamer 36t-4 were 100 nM and 500 nM, respectively. Peak 1 is the complex of (receptor α)-(PDGF-AB)-(aptamer); peak 2 is the complex of (PDGF-AB)-(aptamer); and peak 3 is due to the unbound fluorescent aptamer.

To achieve high sensitivity, a 5:1 ratio of fluorescent 36t-4 to PDGF-AB was used in the experiment to deplete the unbound PDGF-AB, reducing the possible formation of (receptor α)-(PDGF-AB) complex without the fluorescent aptamer. Under the optimum conditions, the method was able to detect at levels as low as 0.5 nM PDGF receptor α as shown in Figure 5.

Figure 6 shows a series of electropherograms from the analysis of PDGF receptor α in mixture solutions containing varying concentrations of receptor α and constant concentrations of PDGF-AB and aptamer 36t-4. A linear calibration curve was obtained for the determination of receptor α with an r^2 value of 0.989. A linear dynamic range from 1 to 50 nM was obtained, and this dynamic range could be extended to higher concentrations of protein by using proportionally higher concentrations of PDGF-AB and 36t-4. The spiked receptor α was also successfully detected in a 10-fold diluted serum as shown in Figure 7. The addition of the nonspecific, nonfluorescent oligonucleotide (49 mer) eliminated the interference from the nonspecific binding of serum proteins to the fluorescent aptamers.

Competitive Assay for PDGF Receptor *β*. Since PDGF receptor *β*, aptamer 20t-4, and aptamer 36t-4 bind only to the PDGF B chain, two possible strategies were considered to detect PDGF receptor *β*. If the binding of PDGF receptor *β* and the aptamer to the PDGF-B chain were compatible with each other, PDGF-BB could be used as a connector to bring the receptor *β* and fluorescent aptamer together, forming a (receptor *β*)-(PDGF-BB)-(aptamer) complex. Alternatively, the analysis of receptor *β* could be carried out in a competitive

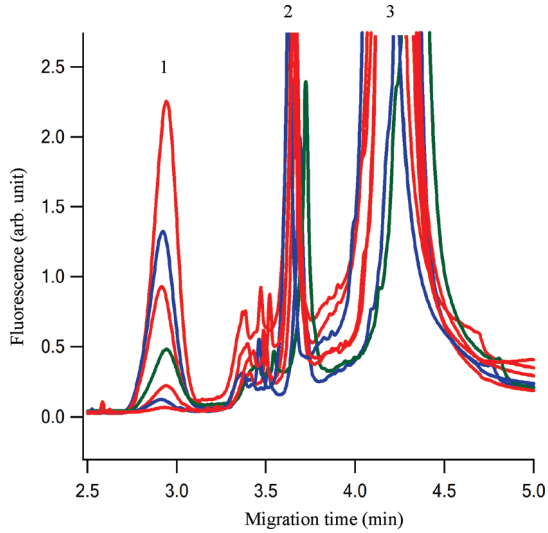

Figure 6. Electropherograms showing the determination of PDGF receptor α. The concentrations of PDGF receptor α were 1, 5, 10, 15, 25, 35, and 50 nM, respectively, from the bottom to the top traces. Peak 1 is the complex of (receptor α)-(PDGF-AB)-(aptamer); peak 2 is the complex of (PDGF-AB)-(aptamer); and peak 3 is due to the unbound fluorescent aptamer.

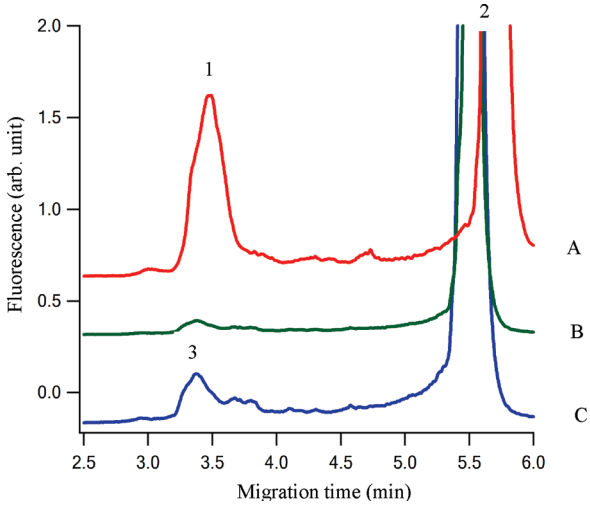

Figure 7. Electropherograms showing the analysis of PDGF receptor α in the diluted human serum sample. All three samples contained 10-fold diluted human serum and 500 nM fluorescently labeled aptamer 36t-4. In addition, sample A contained 30 nM receptor α, 100 nM PDGF-AB, and 5 μM nonspecific oligonucleotide (49 mer); sample B contained 100 nM PDGF-AB and 5 μM nonspecific oligonucleotide. Peak 1 is the complex of (receptor α)-(PDGF-AB)-(aptamer); peak 2 is the unbound fluorescent aptamer; and peak 3 is due to nonspecific binding of serum proteins to the fluorescent aptamer.

assay format based on the competition between the receptor β and a fluorescent aptamer in binding to the PDGF-B chain.

The possibility of forming the (receptor β)-(PDGF-BB)-(aptamer) complex was first determined by mixing receptor β and PDGF-BB with either aptamer 20t-4 or 36t-4. The electropherograms are shown in Supporting Information Figure S6. Although the complex between PDGF-BB and aptamers is formed as expected (peak 1), no ternary complex peak could be attributed to the binding of receptor β, PDGF-BB, and the aptamer. The lack of ternary complex formaton is consistent with previous reports that these aptamers (20t-4 and 36t-4) could inhibit the binding of PDGF-BB to the receptors.[30]

A competitive assay format was tested, and PDGF-AB and BB were compared as the competitive binding targets for analysis of receptor β. The results are shown in Supporting Information Figure S7. The use of PDGF-BB leads to a better sensitivity than AB, which suggests that the binding of the second aptamer to PDGF-BB is weaker than that of the first aptamer. This is not surprising because the presence of negative charges from the first aptamer repels the binding of the second aptamer. Therefore, PDGF-BB and aptamer 20t-4 were chosen for the analysis of receptor β.

The concentration of the aptamer and PDGF-BB is critical to the analysis of receptor β. Too high a concentration produces an overly large (PDGF-BB)-(aptamer) complex peak, and measuring the small change in peak area produced by the low concentration of receptor β is difficult, limiting the sensitivity of the assay. However, too low a concentration would narrow the dynamic range of the analysis. The optimized concentrations of the aptamer and PDGF-BB were 10 nM and 5 nM, respectively. A detection limit of 3 nM ($S/N = 3$) was obtained for analysis of receptor β under the optimized conditions. A calibration curve, as shown in Supporting Information Figure S8, was obtained for the determination of receptor β with an r^2 value of 0.986. A dynamic range from 5 to 200 nM was determined.

DISCUSSION

The differentiation and detection of PDGF-AB and PDGF-BB isomers were achieved by the tunable aptamer CE. Using an aptamer that forms a stable complex with the B chain but not with the A chain of PDGF, the electrophoretic mobilities of the PDGF isomers were tweaked for their separation. PDGF-AB bound to a single aptamer molecule was resolved well from PDGF-BB bound to two aptamer molecules. Simultaneous determination of sub-nM levels of two isomers was accomplished in a single analysis. To our knowledge, this is the first demonstration of separation and determination of these PDGF isomers in a mixture by using an aptamer as a probe.

PDGF-AB was employed as a connector to bring the receptor α and the fluorescent aptamer into a single complex molecule. The formation of a (receptor α)-(PDGF-AB)-(aptamer) complex enabled the detection of the receptor α in a noncompetitive affinity assay. The method was able to detect as low as 0.5 nM of receptor α. Furthermore, by conducting a competitive assay, the determination of receptor β was demonstrated based on competition between the receptor β and a fluorescent aptamer in binding to the PDGF-B chain. The detection limit of 3 nM was obtained for determination of receptor β.

Two aptamers, 20t-4 and 36t-4, showed different performances in analysis of PDGF isomers and their receptors. The differences between these aptamers in the formation of the (PDGF-AB)-(aptamer) complex and the (receptor α)-(PDGF-AB)-(aptamer) complex indicate that aptamers 20t-4 and 36t-4 bind to different sites of the PDGF-B chain. Aptamer 20t-4 has a stronger ability to inhibit the binding of receptor α to PDGF-

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AB. The method developed in this study provides a potentially new approach to determine the impact of aptamers on the inhibition of the binding of PDGF isomers to their receptors.

PDGF isomers (pI 9.5−10) and other positively charged proteins have a tendency to adsorb on the surface of fused silica capillaries. By binding aptamers to these proteins, the negative charges of the aptamers cover the surface positive charges of the basic proteins, reducing the adsorption problem. Therefore, affinity binding of aptamers to these proteins makes the highly positive charged proteins amenable for analysis by free zone capillary electrophoresis. The use of coated capillaries could further reduce adsorption of proteins and their affinity complexes.

The assay is not limited to the PDGF isomers shown here, and the principle can be extended to separation and detection of other protein isomers or a family of proteins to which aptamers can bind. The throughput of the assay can be further enhanced by using electrophoresis systems with multiple capillaries.[31]

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SUPPORTING INFORMATION AVAILABLE

Figures S1−S8. This material is available free of charge via the Internet at http://pubs.acs.org.

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