Biosensors and Bioelectronics 26 (2011) 4832–4836

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Short communication

Bi-cell surface plasmon resonance detection of aptamer mediated thrombin capture in serum

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ARTICLE INFO

Article history:
Received 13 December 2010
Received in revised form 24 May 2011
Accepted 29 May 2011
Available online 6 June 2011

Keywords:
Thrombin
Serum
Aptamer
Bi-cell surface plasmon resonance spectrometer

ABSTRACT

The serine protease coagulation factor thrombin functions primarily in hemostasis, but is also involved in atherosclerosis, thromboembolic disease, cancer and inflammatory disease. Direct measurement of coagulation proteins including thrombin in plasma samples poses a significant challenge because of lack of specific probes and low thrombin concentrations. In addition, high plasma protein concentrations in samples can result in high backgrounds. These challenges were overcome using a bi-cell surface plasmon resonance (SPR) spectrometer with an immobilized thrombin aptamer to measure thrombin in samples passed through a low volume flow cell. For thrombin in Tris–EDTA buffer, the limit of detection (LOD) was 25 nM. Coefficient of variation (CV) for detection of 50 nM was 12.2% and 12.4% for intra and inter-day measurements respectively. This detection was specific for both thrombin aptamer and for thrombin. Using serum samples spiked with thrombin, the LOD was 50 nM with a linear range of detection from 50 nM to 200 nM. However use of serum samples was associated with consistent, low-level background drift. The contributions of nonspecific protein absorption onto the sensor surface and sample flow speed were assessed, and strategies to reduce this background drift were explored. We conclude that the bi-cell SPR platform with an aptamer capture probe can be employed as a highly sensitive real-time, label-free biosensor for the detection of coagulation factors in plasma samples.

1. Introduction

The serine protease thrombin functions as a coagulation factor. Apart from its major function in hemostasis, it is also involved in many pathological disease conditions. Atherosclerosis and thromboembolic disease are the two common diseases associated with an increase in serum thrombin level. The latter has various clinical manifestations including deep venous thrombosis, pulmonary embolism and disseminated intravascular coagulation. It is also observed that an increase in thrombin generation in tumor procoagulation activities may activate platelets, and thus induce angiogenesis and tumor growth (Roselli et al., 2003). Currently, thrombin quantitation in plasma relies on measurements of thrombin activity using chromogenic substrates (Stief, 2006). Thrombin levels estimated by thrombin activity can be falsely elevated because some non-thrombin serum proteases also hydrolyze the chromogenic substrates used in this assay. A direct assay method which measures the amount of thrombin present in samples may yield more reliable thrombin levels for clinical assessments.

Development of direct assays for thrombin has been hindered by the lack of available antibodies against thrombin to serve as probes for immunoassays. However, a novel nucleic acid based probe termed an aptamer specifically binds thrombin and has been used as a capture probe to detect thrombin (Bini et al., 2007; Bock et al., 1992). The thrombin aptamer is a single-strand 15-mer oligodeoxynucleotide that possesses a specific three-dimensional structure in solution, binds to the fibrinogen recognition exosite (exosite 1) of thrombin with nanomolar K_D, and inhibits coagulation (Griffin et al., 1993; Schultze et al., 1994; Tsiang et al., 1995). The thrombin aptamer has been employed in the detection of thrombin upon multiple sensor platforms, including electrocatalytic, electrochemical, piezoelectric, quartz microbalance, and fiberoptic methods (Baldrich et al., 2004; Bini et al., 2007; Centi et al., 2007; Di Giusto et al., 2005; Hianik et al., 2005; Lee and Walt, 2000; Polsky et al., 2006).

SPR is widely used for high-sensitivity, label-free, real-time detection of various biomolecules (Campagnolo et al., 2004;

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doi:10.1016/j.bios.2011.05.049

Fitzpatrick and O'Kennedy, 2004; Jongerius-Gortemaker et al., 2002; Oh et al., 2004; Schasfoort and Tudos, 2008). Analytes such as proteins are detected by measuring the change in the resonance angle for total internal reflection of light that is associated with capture of the analyte on the functionalized surface of the SPR instrument (Liedberg et al., 1983; Piliarik et al., 2009). At the resonance angle, binding of the analyte to its capture probe on the SPR surface changes this angle in proportion to the mass of the analyte captured (Nagata and Handa, 2000). SPR instruments have various mechanisms for measuring the change in this resonance angle. In 1999 Tao and colleagues reported development of a bi-cell photodetector method for measuring the resonance angle change to quantify analyte capture, which calculate the resonance angle change by dividing the difference in light waves detected by the two photocells, originally aligned such that the point of total internal reflection was focused by a prism at the junction of the two photocells, by the sum of light on the two cells and hence it [(A − B)/(A + B)] equaled zero (Tao et al., 1999; Wang et al., 2001, 2007). Upon capture of the analyte on the SPR surface, the light collected by the two photocells changes such that the ratio is proportional to the change in the resonance angle. This method for measuring the change in the resonance angel is reported to be more sensitive for detection of analytes as well as less susceptible to noise that arises due to ambient light variation and laser intensity fluctuations. However, the detection limit of Bioacore instruments are reported to be one to two orders of magnitude greater sensitivity than bi-cell SPR (Mannelli et al., 2005; Wang et al., 2004). Detection limit and precision of bi-cell SPR platform might be enhanced using variations in the analyte capture method including increasing the spacer of the oligonucleotide probe and using sandwich assay methods (Sun et al., 2007; Tang et al., 2007). In addition, carboxylate dextran modified surfaces have lesser non-specific binding when compared to other surfaces and may enhance the specificity of detection (Yao et al., 2006).

We report the performance of a flow-injector bi-cell SPR instrument constructed to the specifications of the Tao instrument for measurement of thrombin in clinically relevant samples. Tao and colleagues had previously demonstrated superior sensitivity for the detection of a nucleic acid target in the femtomolar range using a hybridization probe (Song et al., 2002), while another group was able to improve sensitivity for a nucleic acid target to the attomolar range using a bi-cell SPR with a gold nanoparticle sandwich hybridization assay (Yao et al., 2006). However, to our knowledge the bi-cell SPR of Tao's design has not previously been evaluated for detection and quantification of protein targets as reported herein.

2. Materials and methods

2.1. Reagents

The thio linked DNA thrombin aptamer, its complementary sequence, a non-complementary sequence and a thio linked misaptamer (5′ GGT TGG TGT GGT TGG AA/3ThioMC3-D/3′, 5′ TTCCAA CCA CACCAA CC 3′, 5′ AAA CCCAAC CCA ACC CA 3′ and 5′ TGG GTT GGGTTG GGT TT/3ThioMC3-D/3′) were purchased from Integrated DNA Technologies (Coralville, IA). Human α-thrombin and prothrombin were purchased from Enzyme Research Laboratories (South Bend, IN). Optical fluid (LS-5252) from Lightspan (Wareham, MA), Cleland's Reductacryl Reagent from Calbiochem (San Diego, CA), MicroSpin G-25 columns from Amersham Biosciences (Pittsburg, PA), Microcon YM-3 centrifuge filter devices from Millipore (Burlington, MA) and 97% 6-Mercapto-1-hexanol from Sigma Aldrich (St. Louis, MO). Defined fetal bovine serum was from HyClone (South Logan, UT). All other materials for buffers, bovine serum albumin and Tween 20 were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Coating coverslips with gold

Fisherbrand 18 mm × 18 mm BK-7 glass coverslips (Fisher Scientific) were washed in soap solution, isopropanol and acetone, each for 10 min and then air dried. Coverslips were coated with 2 nm chromium followed by 47 nm of gold, using a Denton Vacuum Desk III bench top sputter coater (Moorestown, NJ).

2.3. Immobilizing aptamer on gold surface

Gold coated coverslips were cleaned in freshly prepared Piranha solution (concentrated sulfuric acid [99.99%] and 30% hydrogen peroxide in the ratio 7:3) for 10 min on a rocker to remove any impurities. The coverslips were then rinsed with double distilled water and ethanol, two times each, and air dried.

The oligonucleotides including the aptamer and the misaptamer were supplied as disulfides. To prepare these for use, the disulfide bonds were reduced with a dithiothrietol (DTT) coated beads (Reductacryl, Calbiochem Inc.). The beads were first resuspended with the oligos in Tris–EDTA (10 mM Tris, 1 mM EDTA, pH 7.5). A ratio of 1 mg oligo with 50 mg resin was used to ensure complete reduction and the suspension was stirred or agitated at room temperature for 15 min. Reductacryl beads were removed using a G-25 Micro Spin Column and centrifuged for 3 min at 735 rcf. The beads stayed in the column, and the oligos (aptamer) were taken in a tube. In order to debuffer the solution, the oligos were added to Microcon YM-3 filter and centrifuged at 14,000 rcf for 30 min. The filtrate was discarded and KH_2PO_4 (1 M, pH 7) was added to the other side of the filter, and again centrifuged at 1000 rcf for 4 min by keeping the filter tube inverted. The solution was collected and made up to 50 μL by adding KH_2PO_4. The 50 μL of purified oligo was coated on the cleaned gold coverslip and incubated for 4 h at room temperature. Aptamers also had two extra bases at the 3′ end, which functioned as a spacer arm. The functionalized surface was then cleaned with double distilled water and air-dried. For control experiments, a 15 mer misaptamer, having identical nucleotide content but not identical sequence, was also immobilized onto gold surfaces as described above. After functionalizing with the probe, the gold surface was further modified by a mercaptohexanol ($SH–(CH_2)_6–OH$) monolayer. For this, 20 mM mercaptohexanol was coated on to the gold for 2 h at room temperature then washed with double distilled water and air dried.

2.4. SPR instrumentation

A prototype of the bi-cell SPR was made by ICx Nomadics Inc. (Stillwater, OK). The specifications of the system have been reported elsewhere (Wang et al., 2007). Briefly, a 4 mW 670 nm diode laser was used as the light source, a BK7 semi cylindrical prism was used in the Kretschmann configuration along with a 4 μL flow cell (with 5 mm^2 sensing area) to hold the sample (Fig. 1A). A bi-cell photo detector was mounted on a linear translation stage for collecting the SPR signal. A differential analog amplifier was used to calculate the sum and differential signal from the bi-cell detector and the signals were passed to an AD converter for data recording with a computer, using LabView 6i (National instruments, Austin, TX).

The aptamer immobilized gold-coated coverslips were placed on to the prism, with a drop of index matching fluid, and the prism was placed on a rotatable holder. An injection flow cell was then secured on top of the prism with screws. The flow cell was connected to a syringe pump that can deliver samples with adjustable flow rate. At the start of the experiment, Tris EDTA buffer was flowed through the flow cell at a rate of 10 μL per min. The laser

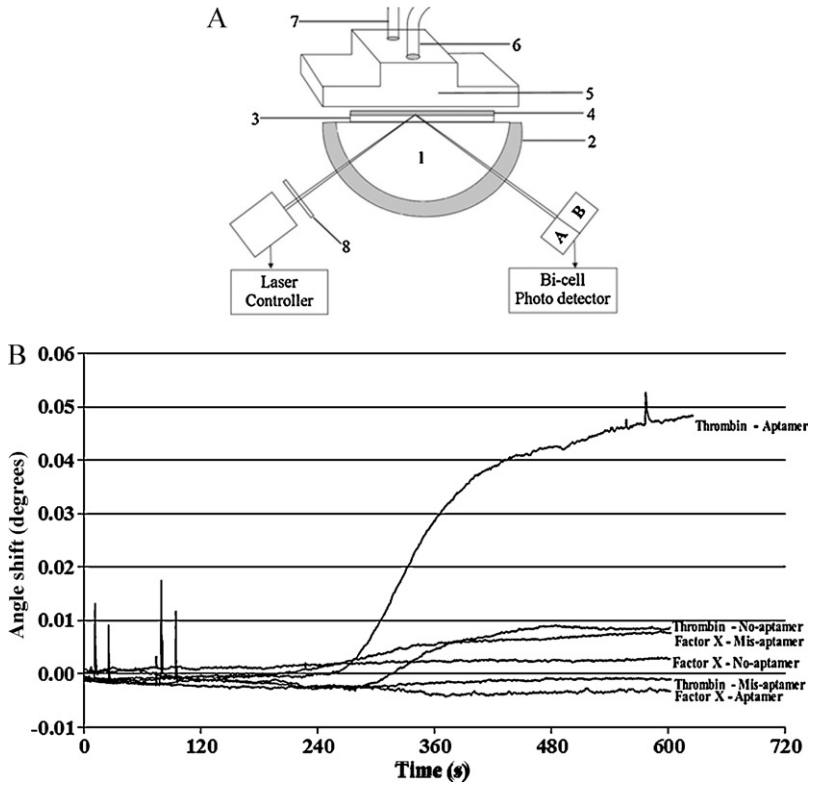

Fig. 1. (A) Schematic diagram of bi-cell SPR setup. 1 – Prism. 2 – Supporting frame for holding prism. 3 – Glass slide. 4 – Metal film. 5 – Supporting frame for Teflon flow cell. 6 – Solution in port. 7 – Solution out port connected to syringe pump. 8 – Lens. (B) Capture of 100 nM thrombin (1 pmol of thrombin/10 μL Tris–EDTA buffer) using the thrombin aptamer. There was no significant angle shift with the controls, which included factor X, capture using mercaptohexanol layer, and misaptamer. Analyte was flowed for 1 min and the detection occurred around 4 min, the time required for the sample to reach the sensor surface. *X*-axis = time in seconds, *Y*-axis = angle shift (degrees).

was then focused on to the prism, and then the prism was rotated until a dark line or dip was seen in the reflected light. Once the dip was clearly focused, all the screws on the prism holder were tightened. The bi-cell photodetector was mounted on a transition stage and the detector was adjusted to the position such that the dip falls in between the two photocells A and B, to make the photo currents from A and B equal.

2.5. Experimental design

Tris–EDTA buffer (Tris 10 mM, EDTA 1 mM, pH 8.0) was flowed through the flow cell at 10 μL per minute. The flow was maintained up to 10–20 min for the drift to settle and a stable base-line was reached. Target molecule in Tris EDTA buffer was passed through the flow cell at the same flow rate. Change in SPR angle shift was calculated using the formula, SPR shift angle = (A − B)/(A + B), which is the ratio of differential sum of the light falling on the two photo cells to the total light falling on the cells. The instrument was calibrated so that one unit of SPR shift corresponds to 1° angle shift. For each experiment, the data was recorded for period of 10 min (except for serum experiments). The resulting data was plotted on an Excel graph. The analytes including α-thrombin, factor X and human prothrombin were thawed at room temperature for 1–2 h. For hybridization experiments using oligonucleotides of sequence complementary and non-complementary sequence, the buffer used was Tris–EDTA with $MgCl_2$ (10 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.6). The same experiment was repeated by using a gold sensor that did not have the aptamer immobilized on it and contained only the mercaptohexanol monolayer. As another negative control, the immobilized misaptamer gold sensor was used to capture both the factor X and thrombin and the SPR angle shift was calculated.

2.6. Reproducibility of experiments

In order to calculate the intra-day and inter-day variations in thrombin capture, three concentrations of thrombin (50, 100 and 200 nM) were used. Data from the three independent experiments were plotted on a graph and coefficient of regression and the coefficient of variation were calculated.

2.7. Capture of human α-thrombin in serum

Defined fetal bovine serum (FBS) was thawed at room temperature and 10% FBS was made in Tris EDTA. The different concentrations of the analyte was spiked in the 10% FBS. For the SPR run we first introduce the 10% FBS through the flow cell at 10 μL per minute for 45 min and then introduced the spiked samples.

3. Results and discussion

3.1. Hybridization experiments

Initial experiments were performed to assess baseline stability and time necessary to collect reliable detection. This was done with hybridization experiments using thrombin aptamer as hybridization probe for its complementary oligonucleotide. Using an inject-flow cell set-up with a total volume of 40 μL (30 μL injector volume ahead of a 10 μL flow cell), a reasonably stable baseline (<0.001° SPR shift angle/min.) was obtained after 10 min at an injector rate of 10 μL/min. This time requirement is attributed to the necessity of the instrument to reach a thermal equilibrium at which time the amount of heat generated by the laser is equal to that dissipated via the exit of the flow sample. Under these conditions, measurement of the change in resonance angle for individual sam-

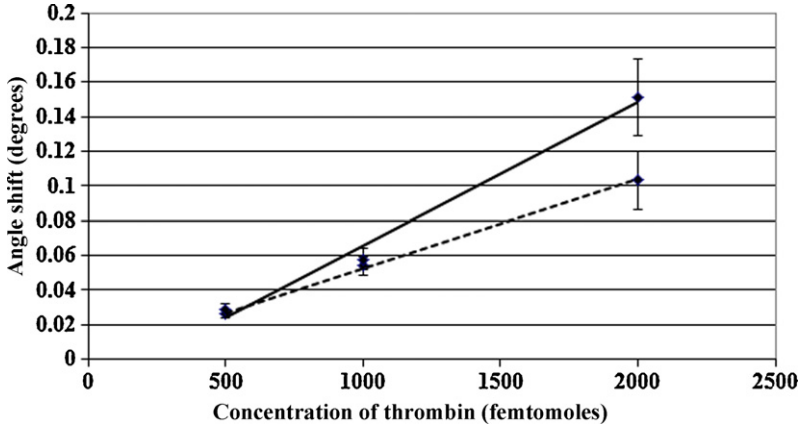

Fig. 2. Dotted line – intra-day variation calculated by doing SPR runs on three different sensor surfaces on the same day. r^2 value = 0.9992. The error bars showing one standard deviation from the mean value of 3 measurements. CV for 500, 1000 and 2000 fmol/10 μL (50 nM, 100 nM and 200 nM) were calculated to be 12.21%, 12.14% and 20.13% respectively. Solid line – inter-day variation calculated by doing SPR runs on three different sensor surfaces on the same day. r^2 value = 0.9892. The error bars showing one standard deviation from the mean value of three measurements. CV for 500, 1000 and 2000 fmol/10 μL (50 nM, 100 nM and 200 nM) were calculated to be 12.43%, 13.09% and 18.6% respectively. *X*-axis = concentration of thrombin in fmol, *Y*-axis = angle shift (degrees).

ples required 4 min, including 3 min for the sample to travel from the injector to the flow cell and 1 min for the SPR signal to reach its plateau. For the hybridization assay, 0.5 pmol of 14mer 4132 Da complementary DNA target in a 10 μL sample (50 nM) was readily detected with a sensorgram demonstrating a plateau in signal within the 1 min injection period with a SPR shift angle of 0.02° (data not shown). Detection was specific for the complementarity of the thrombin aptamer with its target. Using a bi-cell SPR instrument constructed to identical specifications, Song et al. (2002) reported a 0.015° SPR shift angle for 0.28 pmol of a 30mer 14,400 Da DNA target in a 100 μL sample (2.8 nM) with an injector flow that required 7 min to the SPR shift angle data. Taking into account the greater target mass of the 30mer target, the bi-cell SPR instrument used in the experiments reported herein had similar sensitivity to that used by Song and colleagues, which had a reported limit of detection for the 30mer target of 50 fmol.

3.2. Detection of thrombin in buffer

Following optimization of sample analysis, the immobilized thrombin aptamer was used to detect human thrombin in a Tris buffered solution (Fig. 1 B). Detection was specific for both the thrombin aptamer capture probe and for the target analyte thrombin. Substitution of a 14mer oligonucleotide with the same nucleotide GC content, but with a different sequence from the thrombin aptamer or of another serine protease coagulation factor X for thrombin resulted in no change in the SPR shift angle, indicating that the detection of thrombin is specific. However, substitution of 200 nM of human prothrombin for thrombin did result in detection (data not shown); however, prothrombin was not detected when it was present at ≤100 nM. This observation is consistent with the finding that the thrombin aptamer does bind prothrombin but with a lower K_D (50 nM versus 2 nM for thrombin) (Pendergrast et al., 2005), suggesting that the presence of prothrombin in clinical samples may not affect the level of thrombin measured using this technique. A limit of detection (LOD) of 25 nM with a reliable range of detection of 50–200 nM (Fig. 2) was obtained, which was also the range of linearity of SPR shift angle (r^2 = 0.99). This narrow linearity range may be attributable to how the change in resonance angle is measured in the bi-cell SPR instrument, in which once sufficient analyte has been captured to move the point of internal reflection entirely onto one of the two photocells, additional analyte does not result in any further change in the SPR shift angle. The intra- and inter-day coefficients of variation for 50 nM thrombin were 12.2% and 12.4%, respectively. The specificity and sensitivity of thrombin capture using thrombin aptamer on a SPR platform is also dependent on the aptamer immobilization method and the length of the aptamer. Ostatna and colleagues reported higher sensitivity when using biotin–streptavidin based immobilization of the thrombin aptamer as compared to thiolated aptamer (Ostatna et al., 2008). In addition, Tang and coworkers observed enhanced thrombin binding capacity when a 35-mer spacer was added at the 5′ end of the thrombin aptamer (Tang et al., 2007).

3.3. Detection of thrombin in serum

Lastly, the bi-cell SPR instrument was evaluated for measurement of thrombin in a clinically relevant sample containing serum proteins. Detection of thrombin directly in undiluted serum was not practical owing to a stable, but progressively increasing SPR shift angle for serum in the absence of added thrombin. Dilution of the serum to 10% in Tris buffer reduced the magnitude of the progressive drift, but did not eliminate it. One hundred nM thrombin spiked in 10% fetal bovine serum was detected using the same bi-cell SPR instrument (Fig. 3), and the LOD for thrombin detection in serum was 50 nM. Although thrombin levels under pathologic conditions are not measured directly, based on endogenous thrombin potential measurements, the thrombin concentration in these conditions are likely greater than the detection range reported herein for the bi-cell SPR (Hemker et al., 2006). However, the normal human plasma basal concentration of thrombin is likely two to three orders of magnitude lower than the LOD (Mann et al., 2003).

The shape of the capture curve for thrombin in serum may have been changed from that for thrombin in buffer by the drift associated with the presence of serum. This drift may be due to non-specific absorption of serum proteins onto the sensor surface. Neither pre-blocking the sensor surface with bovine serum albumin or by including 0.05% Tween 20 in the sample diminished this drift. Non-specific absorption of serum on sensing surface is a known difficult issue to resolve. Recent report found that use of polyethylene glycol terminated thiol for gold surface blocking can reduce serum non-specific binding dramatically (Gobi et al., 2007). Polonschii et al. reported detection of 10 nM thrombin in 0.25% plasma using thrombin aptamer capture assay on Biacore 3000 SPR instrument (Polonschii et al., 2010). The linear range for this assay appears to be similar to that reported herein. The reduced non-specific absorption of plasma proteins in Polonschii et al. study may be due to the higher dilution of the plasma. The interference from non-specific

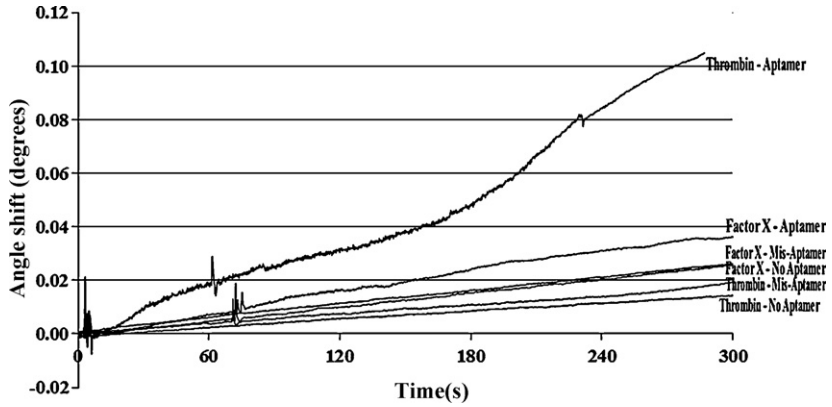

Fig. 3. Capture of 100 nM thrombin in serum (1 pmol thrombin/10 μL of 10% serum) using the thrombin aptamer, detected just around 4 min. There was no significant SPR shift with the controls, which included factor X and capture using misaptamer. Analyte was flowed for 1 min and the detection occurred around 4 min, the time required for the sample to reach the sensor surface. The SPR run was recorded for 5 min. *X*-axis = time in seconds, *Y*-axis = SPR shift angle (degrees).

binding of serum proteins in the current study might be reduced by further diluting the serum or by using a reference channel to cancel out the non-specific binding.

4. Conclusion

Our experimental objective was to assess a bi-cell SPR instrument as a model system for the use of aptamers as capture probes for SPR in diagnostic testing. We were able to show that an aptamer could be used as a capture probe in a bi-cell SPR instrument with high sensitivity and specificity of detection. This real-time label-free biosensor model can be easily adapted to a clinical setting and is much less costly than the commercially available instruments. This model has a great potential to be used for the detection of various biomolecules in a clinical setting.

Acknowledgments

Funds for the experimentation presented herein were from the Center for Veterinary Health Sciences, Oklahoma State University. The bi-cell SPR instrument was supplied by ICx Nomadics Inc, Stillwater, OK.

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