ssDNA Aptamer-Based Surface Plasmon Resonance Biosensor for the Detection of Retinol Binding Protein 4 for the Early Diagnosis of Type 2 Diabetes

ARTICLE *in* ANALYTICAL CHEMISTRY · MAY 2008

Impact Factor: 5.64 · DOI: 10.1021/ac800050a · Source: PubMed

6 AUTHORS, INCLUDING:

Su Jin Lee
Lund University
11 PUBLICATIONS **327** CITATIONS
SEE PROFILE

Javed H. Niazi
Sabanci University
44 PUBLICATIONS **657** CITATIONS
SEE PROFILE

Man Bock Gu
Korea University
199 PUBLICATIONS **4,345** CITATIONS
SEE PROFILE

Anal. Chem. **2008,** *80,* 2867−2873

ssDNA Aptamer-Based Surface Plasmon Resonance Biosensor for the Detection of Retinol Binding Protein 4 for the Early Diagnosis of Type 2 Diabetes

Su Jin Lee,† Byung-Soo Youn,‡ Ji Woo Park,‡ Javed H. Niazi,† Yeon Seok Kim,† and Man Bock Gu*,†

School of Life Sciences and Biotechnology, Korea University, and AdipoGen, Incorporated, Room 641-B, College of Life Science and Biotechnology, Korea University, Anam-Dong, Seongbuk-Gu, Seoul 136-701, Korea

Retinol binding protein 4 (RBP4) is a useful biomarker in the diagnosis of type 2 diabetes since its level in the serum is higher in insulin-resistant states. Accurate measurement of the serum RBP4 levels is hampered by conventional immunologic methods, such as enzyme-linked immunosorbent assay (ELISA). In this study, therefore, we have developed an aptamer-based surface plasmon resonance (SPR) biosensor that can be used to sense for RBP4 in serum samples. A single-stranded DNA (ssDNA) aptamer that showed high affinity (K_d = 0.2 ± 0.03 μM) and specificity to RBP4 was selected. This RBP4-specific aptamer was immobilized on a gold chip and used in a label-free RBP4 detection using SPR. Analysis of RBP4 in artificial serum using SPR was compared with ELISA and Western blot analysis. Our results indicated that the RBP4-specific aptamer-based SPR biosensor gave better dose-dependent responses and was more sensitive than ELISA assays. As such, this RBP4 aptamer-based SPR biosensor can be potentially used to monitor the RBP4 levels within the serum as an indicator of type 2 diabetes.

Obesity and type 2 diabetes are widespread diseases in the world, and the number of patients who suffer from these diseases is increasing.[1−3] Type 2 diabetes occurs as a result of obesity and metabolic syndrome, which eventually leads to insulin resistance.[4,5] Studies showed that adipokines secreted from adipocytes can effect insulin's action.[6] One such serum protein, called retinol binding protein 4 (RBP4), has been linked to obesity-induced insulin resistance and type 2 diabetes.[5] In the adipokine-related type 2 diabetes, the serum level of RBP4 was shown to increase with a concomitant reduction of GLUT4 (glucose transporter) expression, and the elevation of serum RBP4 levels causes systemic insulin resistance.[5,7,8] Several studies have also reported on the relationship between the RBP4 level and type 2 diabetes.[4,9−11] The serum RBP4 is, therefore, a useful biomarker that may enable doctors or medical staff to diagnose insulin resistance or be used for the early monitoring of type 2 diabetes in suspected subjects.[7,12,13]

Conventional methods to detect RBP4 in serum have been reported to have several shortcomings, including with their dynamic range and the protein sequences that were used to generate antibodies for antibody-based assays such as ELISA (sandwich/competitive).[12] However, the most reliable and superior method for assaying serum RBP4 level correlating to insulin resistance is through Western blot analysis.[12] This method, however, is not rapid, requires antibodies, and therefore may not be suitable for a large number of samples that need to be analyzed.

A suitable nonimmunoassay method for the detection of target molecules can be through the selection of a specific aptamer that binds to the target. Aptamers are an emerging class of molecules made of single-stranded DNA (ssDNA) or RNA that rival antibodies in both therapeutic and diagnostic applications[14] since they bind to their specific target molecules with a high specificity and affinity.[15,16] In fact, aptamers for a variety of target molecules have been selected for diverse purposes, including for organic dyes,[17]

* Corresponding author. E-mail: mbgu@korea.ac.kr.
† School of Life Sciences and Biotechnology.
‡ AdipoGen, Inc.

(1) Seidell, J. C. *Horm. Metab. Res.* **1997**, *29*, 155−158.
(2) Seidell, J. C. *Br. J. Nutr.* **2000**, *83* (Suppl. 1), S5−S8.
(3) Engelgau, M. M.; Geiss, L. S.; Saaddine, J. B.; Boyle, J. P.; Benjamin, S. M.; Gregg, E. W.; Tierney, E. F.; Rios-Burrows, N.; Mokdad, A. H.; Ford, E. S.; Imperatore, G.; Narayan, K. M. *Ann. Intern. Med.* **2004**, *140*, 945−950.
(4) Wolf, G. *Nutr. Rev.* **2007**, *65*, 251−256.
(5) Yang, Q.; Graham, T. E.; Mody, N.; Preitner, F.; Peroni, O. D.; Zabolotny, J. M.; Kotani, K.; Quadro, L.; Kahn, B. B. *Nature* **2005**, *436*, 356−362.
(6) Muoio, D. M.; Newgard, C. B. *Nature* **2005**, *436*, 337−338.
(7) Polonsky, K. S. *N. Engl. J. Med.* **2006**, *354*, 2596−2598.
(8) Graham, T. E.; Yang, Q.; Bluher, M.; Hammarstedt, A.; Ciaraldi, T. P.; Henry, R. R.; Wason, C. J.; Oberbach, A.; Jansson, P. A.; Smith, U.; Kahn, B. B. *N. Engl. J. Med.* **2006**, *354*, 2552−2563.
(9) Cho, Y. M.; Youn, B. S.; Lee, H.; Lee, N.; Min, S. S.; Kwak, S. H.; Lee, H. K.; Park, K. S. *Diabetes Care* **2006**, *29*, 2457−2461.
(10) Kovacs, P.; Geyer, M.; Berndt, J.; Kloting, N.; Graham, T. E.; Bottcher, Y.; Enigk, B.; Tonjes, A.; Schleinitz, D.; Schon, M. R.; Kahn, B. B.; Bluher, M.; Stumvoll, M. *Diabetes* **2007**, *56*, 3095−3100.
(11) Takebayashi, K.; Suetsugu, M.; Wakabayashi, S.; Aso, Y.; Inukai, T. *J. Clin. Endocrinol. Metab.* **2007**, *92*, 2712−2719.
(12) Graham, T. E.; Wason, C. J.; Bluher, M.; Kahn, B. B. *Diabetologia* **2007**, *50*, 814−823.
(13) Kloting, N.; Graham, T. E.; Berndt, J.; Kralisch, S.; Kovacs, P.; Wason, C. J.; Fasshauer, M.; Schon, M. R.; Stumvoll, M.; Bluher, M.; Kahn, B. B. *Cell Metab.* **2007**, *6*, 79−87.
(14) Jayasena, S. D. *Clin. Chem.* **1999**, *45*, 1628−1650.
(15) Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505−510.
(16) Tombelli, S.; Minunni, M.; Mascini, M. *Biosens. Bioelectron.* **2005**, *20*, 2424−2434.
(17) Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818−822.

10.1021/ac800050a CCC: $40.75
Published on Web 03/07/2008

antibiotics,[18,19] drugs,[20] peptide or protein targets,[21−23] and those for the detection of disease markers.[24]

Several biosensor systems based on nucleic acids that bind to specific biomolecules or chemicals have been developed and widely studied.[25−27] Studies on biomolecular interactions using surface plasmon resonance (SPR)-based sensing systems including antigen−antibody and protein−DNA interactions have been reported.[28,29] The advantages of SPR-based sensing methods are that they are rapid and offer a label-free detection and real-time monitoring of various binding reactions.[30] Several studies used SPR to measure kinetic parameters, such as the binding and dissociation constants, during an interaction between the ligands and immobilized receptors.[31,32] Moreover, SPR has also been used to confirm the specificity of the receptor for a certain target.[33] Therefore, SPR analysis is a versatile technique, which, in combination with a specific DNA/RNA aptamer for a particular target, can serve as a good biosensor system.

This study involves the selection of an aptamer that specifically bind to RBP4 and an evaluation of its use as an analytical tool for the detection and analysis of RBP4 in artificial serum samples using an aptamer-functionalized gold chip with SPR. Furthermore, the sensitivity of the aptamer-based SPR analysis in detecting RBP4 was compared to the well-established methods, such as ELISA and Western blotting. Our results show that an aptamer-based SPR analysis enabled the specific detection of RBP4 in artificial serum without interference from other adipokines with a large dynamic range as compared to ELISA assays.

MATERIALS AND METHODS

Proteins. The RBP4, adiponectin, and visfatin were provided by AdipoGen, Inc. (Seoul, Korea). Bovine serum albumin (BSA) and human serum albumin (HSA) was purchased from the Sigma Co., and streptavidin was purchased from Fluka.

Immobilization of the Proteins on Tosyl-Activated Magnetic Beads for Aptamer Selection. Immobilization of RBP4 was performed using tosyl-activated magnetic beads (Dynabeads M-280, Dynal Biotech, Norway). The coating process involves the covalent coupling of the *p*-toluenesulphonyl (tosyl) groups on the surface of the magnetic beads with the primary amino groups (NH_2) of the proteins.[34,35] The magnetic beads were washed three times with coupling buffer (0.1 M borate buffer, pH 9.5) and separated by magnetic separation on a magnetic stand (Dynal, Norway). For immobilization, 0.1 mg of human RBP4 was dissolved in the coupling buffer and mixed with the same buffer containing 3×10^8 magnetic beads. The mixture was vortexed for 1 min, and the reaction mixture was incubated for 48 h at room temperature with gentle mixing.

The RBP4-coated magnetic beads were separated from the unbound protein after magnetic separation for 2 min, and the supernatant was removed. The concentration of unbound RBP4 in the supernatant was measured with the micro-BCA kit (Pierce, U.S.A.). The RBP4-immobilized beads were washed as described by the manufacturer and stored in PBS buffer, pH 7.4, containing 0.1% BSA at 4 °C until use. Finally, the RBP4-immobilized magnetic beads were aliquoted out into 200 μL samples. Fresh aliquots of the RBP4-coated beads (13.6 $\mu g/6 \times 10^7$ beads) were used for each round of SELEX (systematic evolution of ligands by exponential enrichment). For the counterselection steps, the beads coated with adiponectin (13.4 $\mu g/6 \times 10^7$ beads), visfatin (14.6 $\mu g/6 \times 10^7$ beads), or BSA (71 $\mu g/6 \times 10^7$ beads) were used. Immobilization of these proteins was done in the same manner as with RBP4.

Random DNA Library and Primers. The random oligonucleotide library (3×10^{16} DNA molecules) contained a central random 40-nucleotide region flanked on both sides by 18-nucleotide primer binding regions. The sequences of the random oligonucleotides and primers are as follows: random DNA library (RL): 5′-ATACCAGCTTATTCAATT-N40-AGATAGTAAGTGCAATCT-3′, fluorescence-labeled forward primer (FP): 5′-fluorescein−ATACCAGCTTATTCAATT-3′, nonlabeled forward primer (NP): 5′-ATACCAGCTTATTCAATT-3′, and reverse primer (RP): 5′-AGATTGCACTTACTATCT-3′.[36] All the above oligonucleotides were purchased in pure form (GenoTech Co., Korea).

In Vitro Selection of DNA Aptamers for RBP4. The RBP4-coated magnetic beads were washed eight times with binding buffer (100 mM NaCl, 20 mM Tris−HCl, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, 0.02 Tween-20, pH 7.6) before the start of each SELEX round.[36] The ssDNA library was heated to 90 °C for 10 min, quickly cooled and stored at 4 °C for 15 min, and incubated at 25 °C for 7 min just before the start of the SELEX procedure. During the first round of selection, the RBP4-coated beads were suspended in 500 μL binding buffer containing 3×10^{16} molecules of linearized random DNA pool. This mixture was incubated at room temperature for 30 min with mild shaking, and the unbound oligonucleotides were removed by washing with binding buffer. Elution of the bound oligonucleotides from the RBP4-coated magnetic beads was performed by incubating the bead−DNA complex in 200 μL of elution buffer (40 mM Tris−HCl, 10 mM EDTA, 3.5 M urea, 0.02% Tween-20 pH 8.0) followed by heat treatment at 80 °C for 10 min with mild shaking. The SELEX procedure including elution, separation of double-stranded DNA

(18) Schurer, H.; Buchynskyy, A.; Korn, K.; Famulok, M.; Welzei, P.; Hahn, U. *Biol. Chem.* **2001**, *382*, 479−481.
(19) Tereshko, V.; Skripkin, E.; Patel, D. J. *Chem. Biol.* **2003**, *10*, 175−187.
(20) Windbichler, N.; Schroeder, R. *Nat. Protoc.* **2006**, *1*, 637−640.
(21) Kirby, R.; Cho, E. J.; Gehrke, B.; Bayer, T.; Park, Y. S.; Neikirk, D. P.; McDevitt, J. T.; Ellington, A. D. *Anal. Chem.* **2004**, *76*, 4066−4075.
(22) Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J. *Nature* **1992**, *355*, 564−566.
(23) Centi, S.; Tombelli, S.; Minunni, M.; Mascini, M. *Anal. Chem.* **2007**, *79*, 1466−1473.
(24) Tang, Z.; Shangguan, D.; Wang, K.; Shi, H.; Sefah, K.; Mallikratchy, P.; Chen, H. W.; Li, Y.; Tan, W. *Anal. Chem.* **2007**, *79*, 4900−4907.
(25) Bang, G. S.; Cho, S.; Kim, B. G. *Biosens. Bioelectron.* **2005**, *21*, 863−870.
(26) Tombelli, S.; Minunni, M.; Luzi, E.; Mascini, M. *Bioelectrochemistry* **2005**, *67*, 135−141.
(27) Xiao, Y.; Lubin, A. A.; Heeger, A. J.; Plaxco, K. W. *Angew. Chem., Int. Ed.* **2005**, *44*, 5456−5459.
(28) Homola, J. *Anal. Bioanal. Chem.* **2003**, *377*, 528−539.
(29) Shumaker-Parry, J. S.; Aebersold, R.; Campbell, C. T. *Anal. Chem.* **2004**, *76*, 2071−2082.
(30) Teramura, Y.; Arima, Y.; Iwata, H. *Anal. Biochem.* **2006**, *357*, 208−215.
(31) Win, M. N.; Klein, J. S.; Smolke, C. D. *Nucleic Acids Res.* **2006**, *34*, 5670−5682.
(32) Caelen, I.; Kalman, A.; Wahlstrom, L. *Anal. Chem.* **2004**, *76*, 137−143.
(33) Leontiou, C.; Lightowlers, R.; Lakey, J. H.; Austin, C. A. *FEBS Lett.* **2003**, *554*, 206−210.
(34) Han, F. C.; Luo, J.; Guo, H. F.; Zhang, W. H.; He, D. C.; Yan, X. J. *World J. Microbiol. Biotechnol.* **2007**, *23*, 1041−1045.
(35) von Bernhardi, R.; Ramirez, G.; Matile, H.; Dobeli, H. *Eur. J. Neurosci.* **2001**, *14*, 946−956.
(36) Stoltenburg, R.; Reinemann, C.; Strehlitz, B. *Anal. Bioanal. Chem.* **2005**, *383*, 83−91.

Table 1. Sequences Shown in the Table Are the 40-Nucleotides Variable Regions (N40) from Each 76-Mer ssDNA Aptamer[b]

Aptamer[a]	Random N40 sequences (5' to 3')
No.7	A C G G T G C G G A G G G G G A G G G T G G C G G G T T G T G T C G G T G T G G
No.8	G G G A G C G G G G G G A G G G T G T C A C A G G C G G G T G T G T T G T G G A
No.38	A C A G T A G T G A G G G G T C C G T C G T G G G G T A G T T G G G T C G T G G
No.40	A C G G T G T G G G C A G T C C A G T T C C C A A T G T T G G G G T C G T G G G G
No.41	G G G G G C G G G C G G G T G G C G T T C T A T T T G C G G T G T G T G G G T A
No.43	G G G G C G G C G G G G T G G G G A G C G T G G T G G T G A G G G T G C G G G T C
No.44	G G C G A C G G A C C T G T G A T G T G T G T A T G G C T C A T A G G G G T G T

[a] Aptamers that bind to RBP4 were selected by the systematic evolution of ligands by exponential enrichment (SELEX) method as described in the Materials and Methods, and the enriched aptamers were sequenced. [b] The sequence of each aptamer is 5′-ATACCAGCTTATTCAATT-**N40**-AGATAGTAAGTGCAATCT-3′. Sequences highlighted in gray are the conserved bases. The sequences shown in boxes represent the consensus sequences, and those in bold indicate possible G-quartets. The aptamers shown in the table were selected from the pool of 3×10^{16} random ssDNA molecules after enrichment as described in the Materials and Methods.

(dsDNA) to ssDNA, and polymerase chain reaction (PCR) conditions were followed as described previously.[36]

During enrichment, the ssDNA obtained from the previous round was used as the starting ssDNA pool in the next selection round with fresh RBP4-immobilized magnetic beads. To obtain more specific ssDNA that bind to RBP4, counter selections were performed after the second and ninth rounds. The counter selection was performed with naked magnetic beads and the beads immobilized with either adiponectin, visfatin, or BSA. The concentration of the ss/dsDNA was measured using a Nanodrop (ND-1000 spectrophotometer, Nanodrop Technologies, Inc.). The above procedure was repeated after every SELEX round until the recovery of bound ssDNA was greater than ~90% of the DNA pool that was added.

Cloning, Sequencing, and Analysis of the Selected Aptamers. The enriched ssDNA, which was recovered from the last selection round, was amplified using the NP and RP under the standard conditions as described above. The resulting unlabeled PCR product was column purified (MinElute, Qiagen) and cloned using the pCR2.1-TOPO vector in *Escherichia coli* TOP10 (TOPO TA Cloning Kit, Invitrogen). Positive clones were picked and grown in LB media containing 50 μg/mL ampicillin, and the plasmid DNA was extracted with a MiniPrep kit (Qiagen). The aptamer insert in the plasmid DNA of each clone was sequenced (GenoTech, Korea). The aptamer sequences were analyzed and the secondary structure was predicted by means of the free-energy minimization algorithm according to Zuker[37] using the web server based mfold tool (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi).

Binding Assays and Determination of K_d by Surface Plasmon Resonance. Binding assays and determination of the dissociation constant (K_d) for the selected aptamer were performed using SPR (Eco Chemie, Netherlands). The aptamers' specificity to the target protein, RBP4, was confirmed using other coexisting serum proteins or adipokines, such as adiponectin (ADPN), visfatin (VSF), HSA, and BSA, as negative controls.

For SPR analysis, the aptamer was immobilized on a bare gold chip as follows: A bare gold chip was cleaned by washing it with ethanol and distilled water and immersing it in 50 mM 3,3-dithiopropionic acid solution at room temperature. The chip was subsequently activated with a 0.2 M *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide (EDC) and 0.05 M *N*-hydroxysuccinimide (NHS) (Sigma Co.) solution. The carboxyl-activated surface was incubated with 100 μg/mL streptavidin (Fluka) for 30 min on ice; this resulted in the binding of the streptavidin to the activated carboxyl surface. The unoccupied carboxyl groups were blocked by adding a 20 mM ethanolamine solution.

The biotinylated ssDNA aptamers were immobilized on the streptavidin-coated gold chip. At first, the streptavidin-coated gold chip was incubated with 1 μM biotin-labeled ssDNA solution at room temperature for 40 min. The chip was deactivated (free groups blocked) by the addition of 50 μg/mL BSA solution. SPR analysis using the surface-activated gold chip was performed after the injection of 50 μL of a 1 μM protein (RBP4/ADPN/VSF/BSA/HSA) sample over the sensor chip. The binding reaction was allowed to occur for 30 min with a 5 min dissociation time at 25 °C. The binding reaction for each sample was performed in triplicate. A highly specific aptamer was chosen, and the dissociation constant was determined via the response units (RU) with RBP4 (0−2 μM). To determine the dissociation constant (K_d), the SPR-RU was plotted against the concentration of RBP4 used, and the average data points from triplicates were fitted with a nonlinear regression analysis with the help of the following equation:

$$y = B_{max}(\text{free ssDNA})/K_d + (\text{free ssDNA}) \qquad (1)$$

where y is the degree of saturation, B_{max} is the number of maximum binding sites, and K_d is the dissociation constant.[38]

RBP4 Analysis in Artificial Serum Samples by SPR, ELISA, and Western Blotting. Artificial serum samples were prepared based on the normal serum compositions[39,40] and contained 70 μg/mL RBP4, 16 ng/mL VSF, 30 μg/mL ADPN, and 40 mg/mL HSA in 0.1 M PBS (pH 7.4). The detection of RBP4 in the artificial serum sample was performed by aptamer-based SPR analysis. A gold chip immobilized with 1 μM aptamer (no. 38)

(37) Zuker, M. *Nucleic Acids Res.* **2003**, *31*, 3406−3415.

(38) Muller, M.; Weigand, J. E.; Weichenrieder, O.; Suess, B. *Nucleic Acids Res.* **2006**, *34*, 2607−2617.

(39) Moriwaki, H.; Fukushima, H.; Shiraki, M. *Nippon Rinsho* **2004**, *62* (Suppl. 12), 138−140.

(40) Chen, M. P.; Chung, F. M.; Chang, D. M.; Tsai, J. C.; Huang, H. F.; Shin, S. J.; Lee, Y. J. *J. Clin. Endocrinol. Metab.* **2006**, *91*, 295−299.

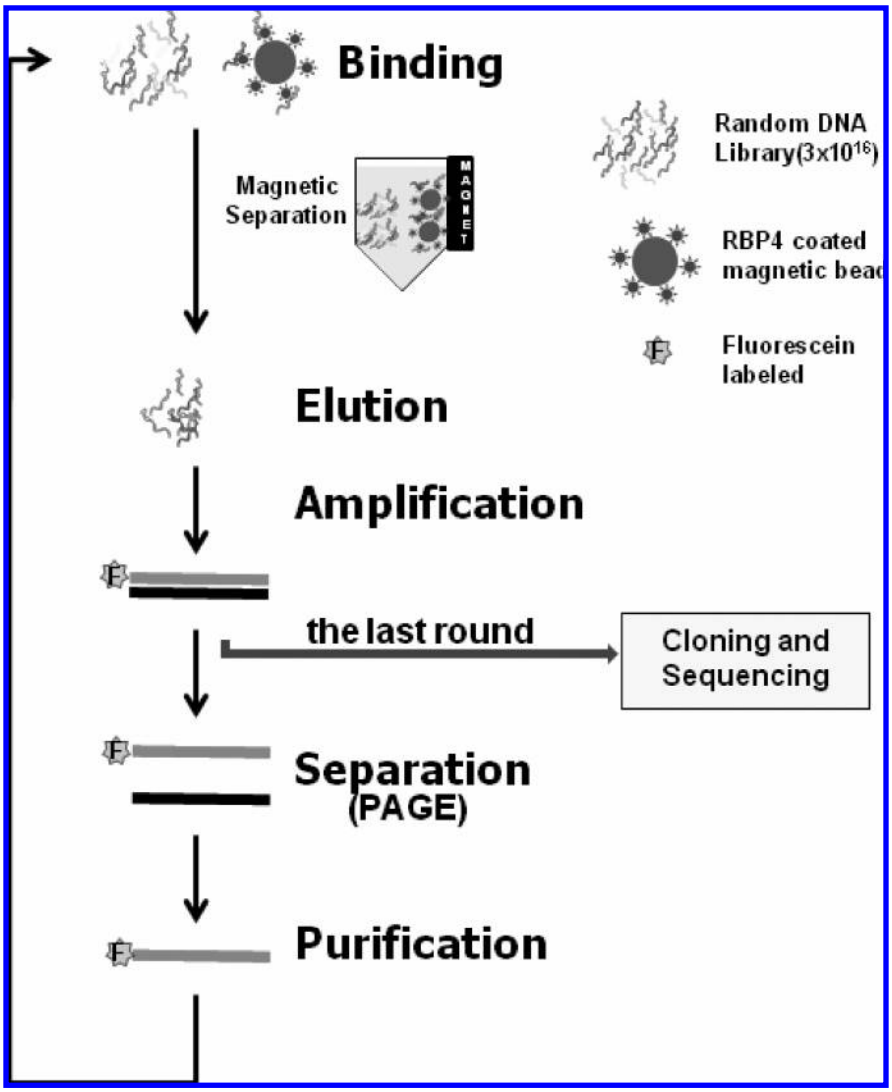

Figure 1. Schematic showing aptamer isolation by the Flu-mag systematic evolution of ligands by exponential enrichment (SELEX) (ref 36).

(Table 1) was used for the RBP4 binding reaction with the help of an SPR detection system. RBP4 detection was done with different dilutions (1:100 to 1: 350) of artificial serum samples. The binding and dissociation conditions were the same as described above, and the reaction was performed in triplicate. All SPR experiments were carried out in a single stretch due to its continuous and stable performance. Hence, the intra-assay coefficient of variation was 1−12%.

The RBP4 concentration in the artificial serum was also measured with a competitive ELISA kit (AdipoGen, Inc.). Artificial serum samples (50 μL) were transferred into the test wells in triplicate. Afterward, 50 μL of anti-RBP4 (AdipoGen, Inc.) was added and incubated at 37 °C for 1 h. The wells were washed three times with PBS containing 0.05% Tween-20. The horseradish peroxidase-conjugated secondary antibody (Jackson Immuno Research, PA) reaction was performed at 37 °C for 1 h, and then the wells were washed five times with PBS containing 0.05% Tween-20. The colorimetric reaction was performed for 20 min with the addition of a 3,3′,5,5′-tetramethylbenzidine (TMB) solution. The optical densities were measured at 450 nm with the help of a microplate reader (Merck, Darmstadt, Germany). The ELISA system had an intra-assay coefficient of variation of 4−8% and an interassay coefficient of variation of 5−10%.

For a more reliable comparison, Western blotting was also performed. The sera was diluted from 1:100 to 1:1200 in 2× sample buffer and subjected to SDS−PAGE on a 12% gel. The proteins were transferred on to nitrocellulose membranes (Amersharm, IL). The primary pAb rabbit polyclonal antibody against human RBP4 was diluted 1:2000 in 5% nonfat dry milk in PBS containing 0.05% Tween-20 (blocking solution), whereas the secondary antibody (horseradish peroxidase-conjugated antirabbit IgG) was diluted 1:5000 in the blocking solution. Target protein bands were detected by chemiluminescence (Pierce Chemical Co., IL), and the band intensities were analyzed using LAS-1000 (Fujifilm, Japan).

RESULTS

Selection of ssDNA Aptamer that Binds to Retinol Binding Protein 4. A ssDNA aptamer that binds to RBP4 was successfully selected from a random ssDNA library of 3×10^{16} molecules using Flu-mag SELEX (Figure 1). The fraction of ssDNA eluted during each selection round increased as the enrichment process

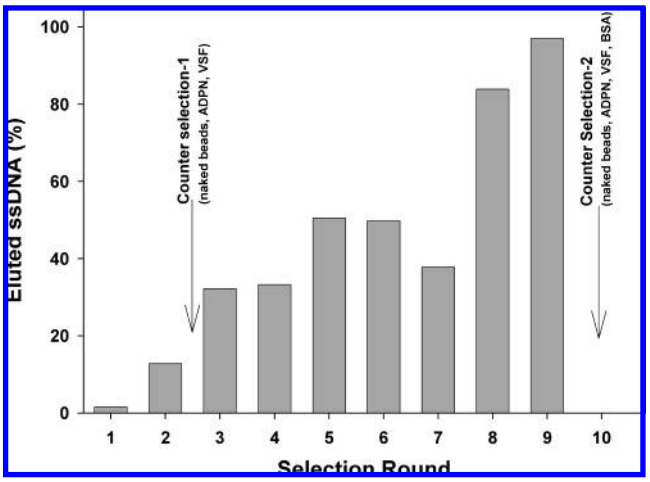

Figure 2. Selection of ssDNA aptamers that bind to RBP4-coated magnetic beads from a random library of 3×10^{16} molecules. The ssDNA recovered after each selection round was calculated as the percent of RBP4-bound ssDNA from the added ssDNA pool. The arrows indicate the counter selection performed using naked tosyl-activated magnetic beads, beads coated with adiponectin (ADPN), visfatin (VSF), and bovine serum albumin (BSA).

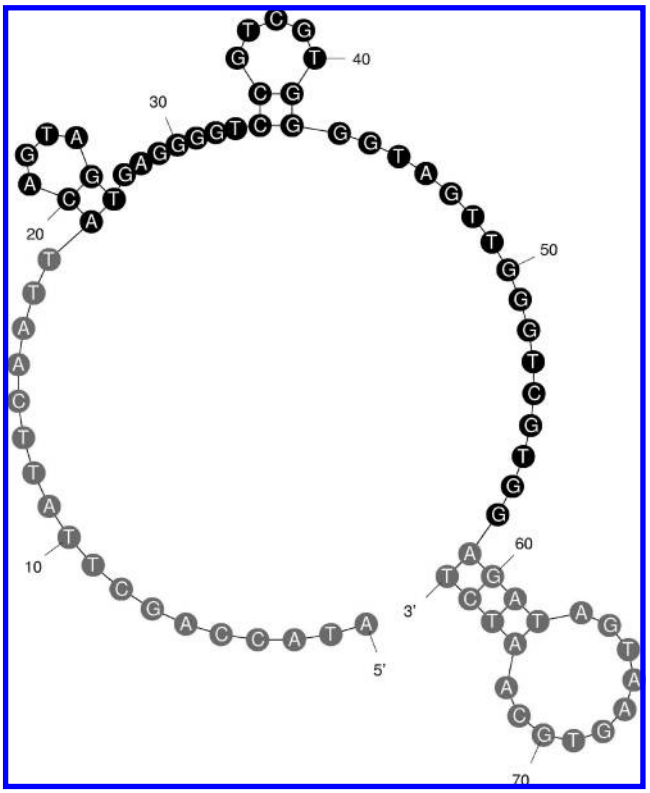

Figure 3. Secondary structure model of no. 38 aptamer as predicted by the mfold program. The aptamer is composed of a 76-mer containing a 40-nucleotide random sequence (highlighted in dark circles) flanked by two 18-nucleotide primer binding regions (gray circles).

progressed (Figure 2). A total of nine selection rounds were performed until the recovery of the ssDNA bound to RBP4 reached more than 90% of the added DNA pool (Figure 2). Aptamers that specifically bind to RBP4 were selected for after the elimination of nonspecific oligonucleotides by counter selection using naked magnetic beads and beads coated with either the ADPN, VSF, or albumin (BSA) protein after the second and last

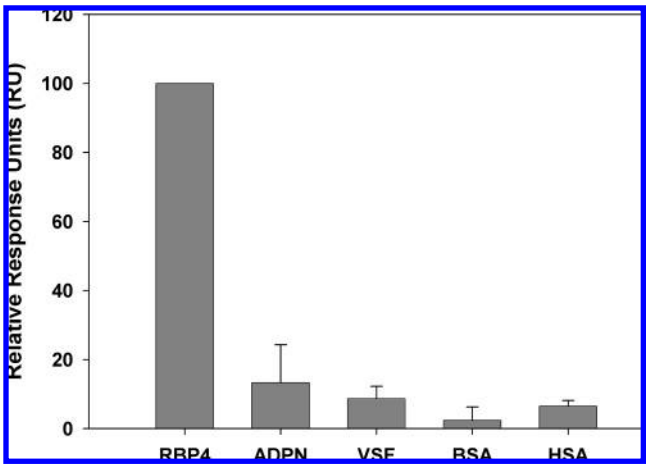

Figure 4. Specificity of aptamer no. 38 to RBP4. Specificity of the aptamer was determined with SPR analysis using a constant amount of ssDNA immobilized on the gold chip and by varying the concentrations of retinol binding protein 4 (RBP4), adiponectin (ADPN), visfatin (VSF), bovine serum albumin (BSA), or human serum albumin (HSA). The response units (RU) were obtained with 1 μM protein solutions at 25 °C and are shown relative with the RBP4 results.

selection rounds (Figure 2). As a result, seven aptamers were selected, and the sequences are shown in Table 1. Four aptamers that had the expected size of 76 nucleotides were selected for further screening. Each contained a central random 40 nucleotides (N40) (Table 1) flanked on both sides by the 18-nucleotides primer binding regions.

Several conserved nucleotides were found within the N40 region of the aptamers and are highlighted in Table 1. For instance, aptamer no. 38 shared a conserved sequence of ACGGT and AGGGG at bases 19−23 and 26−32, respectively, with aptamer no. 7. Furthermore, out of the seven aptamers isolated, nos. 41 and 42 were identical in their sequences. The secondary structure of one of the representative RBP4 aptamers (no. 38) as predicted by the mfold program is depicted in Figure 3. A typical stem and loop motif was seen, which is predicted to be the binding region for the target protein.[41] Furthermore, two G-rich regions were found within the no. 38 aptamer sequence, which are predicted to form the G-quartet structure (Table 1).

Characterization of the RBP4 Aptamer. The binding characteristic of the aptamers with RBP4 was examined by SPR analysis using an aptamer-functionalized gold chip. Initially, the aptamers were biotinylated so as to facilitate immobilization on a streptavidin-coated gold chip. The gold chips were functionalized with different biotinylated aptamers (nos. 7, 38, 40, or 44) for each of the RBP4 binding assays. First, the SPR binding assays were performed using RBP4 as the primary target protein. Consequently, other proteins, such as ADPN, VSF, BSA, and HSA, were used as counter targets (negative controls) to evaluate the aptamers' specificity. The binding of three aptamers (nos. 7, 40, and 44) failed to show specific binding to RBP4 alone and, thus, were not considered for further study. However, tests with aptamer no. 38 with RBP4 showed a relative RU of 100 but gave values of only 13.3, 8.7, 2.4, and 6.5 with ADPN, VSF, BSA, and HSA proteins, respectively (Figure 4). Further, the binding of no.

(41) Lee, S. K.; Park, M. W.; Yang, E. G.; Yu, J.; Jeong, S. *Biochem. Biophys. Res. Commun.* **2005**, *327*, 294−299.

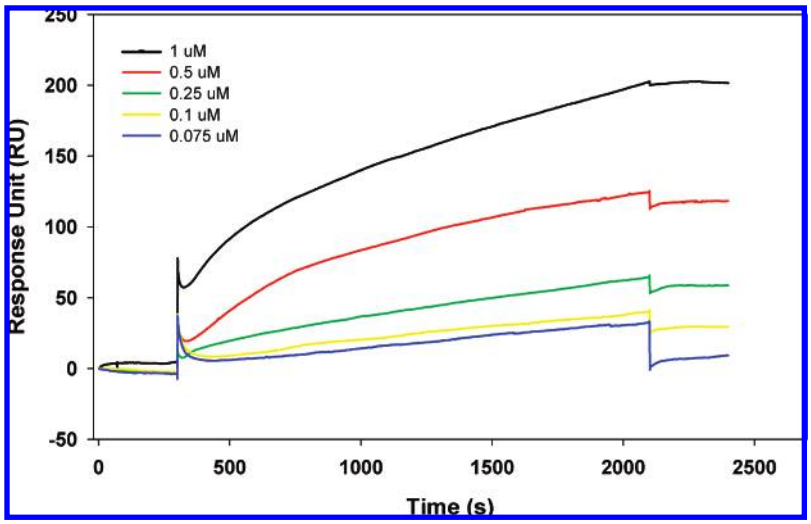

Figure 5. Concentration-dependent surface plasmon resonance (SPR) biosensor response to RBP4. The binding reaction was performed by SPR analysis using aptamer no. 38 and various concentrations of RBP4 (0.075−1.0 μM) at 25 °C.

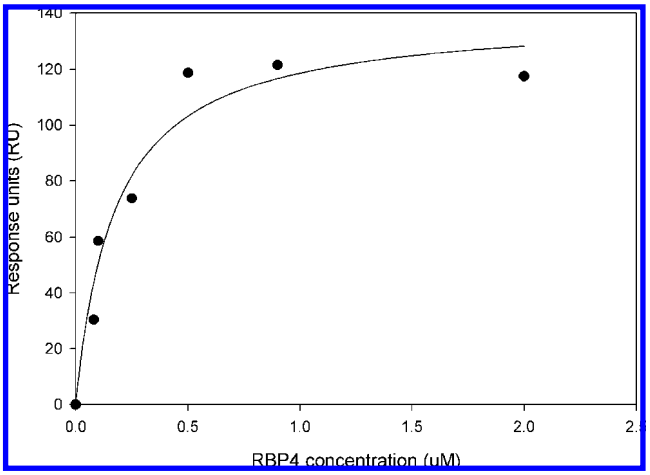

Figure 6. Characterization of the RBP4 binding by aptamer no. 38. SPR analysis was performed using a constant amount of ssDNA immobilized on the gold chip but with a varying amounts of RBP4 (50 μL) at 25 °C. The saturation curve was obtained by plotting the response units (RU) as a function of RBP4 concentration using average values from three independent determinations. The dissociation constant (K_d) was calculated by nonlinear regression analysis.

38 aptamer to RBP4 was dose-dependent (Figure 5), and the minimum detection limit in SPR biosensor using no. 38 aptamer was determined to be 75 nM (1.58 μg/mL) RBP4.

The dissociation constant (K_d value) was determined by SPR analysis using a constant amount of aptamer (no. 38) immobilized on the gold chip but a varied concentration of RBP4 in the sample (0.075−2 μM). From these results, a saturation curve was obtained (Figure 6), and the corresponding K_d value was determined to be 0.201 ± 0.029 μM using eq 1. It is clear that aptamer no. 38 specifically binds to RBP4 with a high affinity and, therefore, can serve as a good candidate for sensor applications.

RBP4 Analysis by Aptamer-Based SPR, Competitive ELISA, and Western Blotting. To utilize this aptamer to sense for RBP4 in serum samples, it was necessary to test aptamer no. 38's ability to discriminate for RBP4 from other serum proteins. For this, the aptamer's efficiency to specifically bind RBP4 was analyzed in an artificial serum sample using SPR. The sensitivity of RBP4 detection in the artificial serum sample had a linear range at various dilutions (150−350×) (Figure 7A). The dynamic range of detection with the aptamer was dose-dependent and sensitive up to and including a 350× dilution, which was more sensitive than competitive ELISA, in which the linear range was seen with only lower dilutions (100−250×) (Figure 7, parts A and B). In contrast, detection of RBP4 using Western blotting was more sensitive than either SPR or ELISA and had a broad linearity (100−1000×) (Figure 7C). However, both ELISA and Western blotting require antibodies for analysis, whereas SPR can be performed using chemically synthesized DNA aptamers.

DISCUSSION

Retinol binding protein 4 is one adipokine responsible for obesity-induced insulin resistance and, thus, a potential therapeutic target in type 2 diabetes.[42] Therefore, RBP4 may be a useful biomarker for the diagnosis of insulin resistance and as an indicator for the risk of type 2 diabetes.[5,8] There are many examples where important biological markers of biochemical processes are present which can be detected using conventional immunoassays, including serum RBP4. However, these conventional methods for detection and measurement, such as ELISA, have several shortcomings,[12] including assay saturation, poor linearity of dilution, limited assay utility, and an immunoreactivity with nontarget molecules.[12] To resolve such limitations, alternative approaches to improving the sensitivity are in demand. Only Western blotting provides results with a greater dynamic range and less overlap between the control and test samples. But this method is laborious and requires antibodies and so is not suitable for the analysis of a large number of specimens.

Single-stranded DNA or RNA aptamers that bind to specific target molecules have been used for biosensing as a substitute for antibodies in bioanalytical sensing and several advantages over immunosensors were found: (a) in vivo immunization of animals to obtain antibodies is eliminated, (b) they can be chemically synthesized unlike antibodies, (c) no structural perturbation of aptamers occurs, and (d) they are thermally stable.[43] Aptamers

(42) Tamori, Y.; Sakaue, H.; Kasuga, M. *Nat. Med.* **2006**, *12*, 30−31; discussion 31.
(43) Willner, I.; Zayats, M. *Angew. Chem., Int. Ed.* **2007**, *46*, 6408−6418.

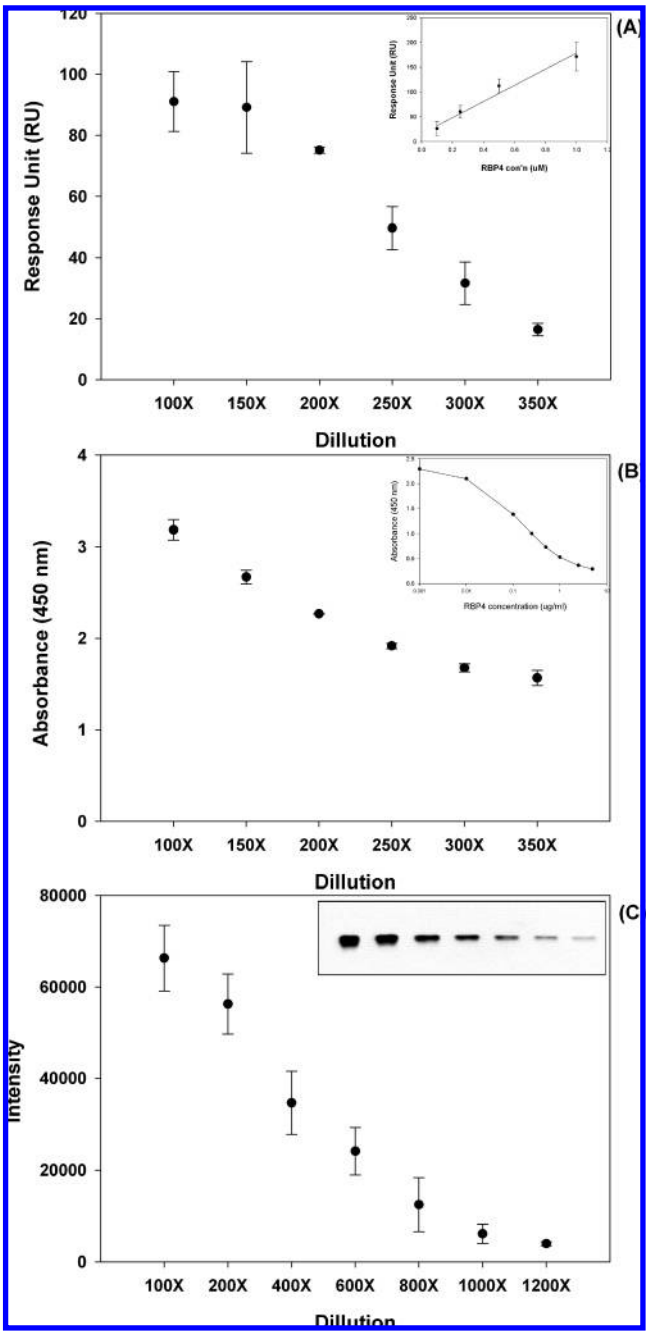

Figure 7. Detection of RBP4 in artificial serum. (A) Aptamer-based SPR biosensor showing the dose-dependent response to RBP4 in serum. The inset shows the dose-dependent SPR signal from the RBP4 standards. (B) Competitive enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of RBP4. The inset shows the standard plot used. (C) Western blot analysis. The inset shows the Western blot results used to calculate the intensities.

are generally isolated from combinatorial libraries of synthetic nucleic acids by an iterative process of reamplification of the nucleotide (SELEX). In recent years, many aptamers (DNA/RNA) have been developed and applied in the detection and analysis of a variety of targets.

In this study, we report a novel ssDNA aptamer (no. 38) that specifically binds to RBP4 with a high specificity and affinity (K_d of 0.2 ± 0.03 μM) (Figures 4 and 6). To our knowledge, this is the first aptamer to be reported on that is specific for full-length human RBP4, and there are no sequence variants of full-length RBP4 found in human serum. However, partially degraded RBP4 has been reported to be detected in the urine, but it was not a sequence variant of full-length RBP4.[12] Furthermore, this aptamer was applied as the bioreceptor component within SPR, and thus, we developed a label-free aptamer-based SPR biosensor for the detection and analysis of RBP4. The sensitivity of aptamer-based SPR biosensor was compared to well-established methods, such as competition ELISA[44] and Western blotting using artificial serum samples. Our results show that the aptamer-based SPR biosensor was more sensitive than ELISA (Figure 7, parts A and B). This was illustrated by the inability of competitive ELISA to distinguish between the samples when a higher dilution of the serum sample was used (Figure 7B), but the aptamer-based SPR biosensor was more sensitive (Figure 7A). However, the Western blot analysis was more sensitive than either of these methods. Although not as sensitive as the Western blot analysis, the minimum detection limit with the aptamer-based SPR biosensor was 75 nM (1.58 μg/mL) RBP4, which is sufficiently sensitive to probe for RBP4 in serum from people at risk for type 2 diabetes. For instance, the normal range of RBP4 in the Japanese population has been shown to be 24−79 μg/mL,[39] whereas it is increased by around 71% in individuals facing the risk of insulin resistance and type 2 diabetes.[4] In addition, typical concentration of ADPN and VSF in human serum has been reported to be 30 μg/mL and 16 ng/mL, respectively,[39,40] and the same concentration was used to make artificial serum samples, which did not interfere in RBP4 detection in this study.

CONCLUSION

The higher RBP4 levels in serum may contribute to the onset of obesity or type 2 diabetes, and these levels can be determined and used as a potential indicator of health risks. We report a novel method by which the binding of an aptamer to RBP4 can be determined using an SPR detection system. The RBP4-specific ssDNA aptamer (no. 38) was composed of 76 bases, with a random 40-nucleotide central region, which binds to RBP4 with high affinity ($K_d = 0.2 \pm 0.03$ μM). With the use of a mixture of other serum proteins, such as ADPN, VSF, and HSA, present in an artificial serum sample, it was demonstrated that it selectively binds to RBP4. The efficiency and sensitivity of RBP4 analysis with SPR was compared to conventional antibody-based methods, such as ELISA and Western blotting. Our studies demonstrated that the aptamer-based SPR biosensor was more sensitive than the ELISA assays. Because of the fact that the SPR biosensor developed in this study is a non-antibody-based detection system, it offers many advantages over antibody-based methods, thus enabling a label-free, rapid, reusable, and sensitive method for the detection of RBP4 in serum.

ACKNOWLEDGMENT

This work was supported in part by a Grant from the Seoul R&BD Program (10582 to B.-S. Youn).

Received for review January 9, 2008. Accepted February 6, 2008.

AC800050A

(44) Ferreira, C. S.; Matthews, C. S.; Missailidis, S. *Tumor Biol.* **2006**, *27*, 289−301.