Aptamer-Linked Assay for Thrombin Using Gold Nanoparticle Amplification and Inductively Coupled Plasma–Mass Spectrometry Detection

ARTICLE *in* ANALYTICAL CHEMISTRY · SEPTEMBER 2009

Impact Factor: 5.64 · DOI: 10.1021/ac900961y · Source: PubMed

5 AUTHORS, INCLUDING:

Qiang Zhao

25 PUBLICATIONS **502** CITATIONS

SEE PROFILE

Chun-Gang Yuan

North China Electric Power University

19 PUBLICATIONS **766** CITATIONS

SEE PROFILE

Xing-Fang Li

University of Alberta

123 PUBLICATIONS **3,128** CITATIONS

SEE PROFILE

X. Chris Le

University of Alberta

248 PUBLICATIONS **7,753** CITATIONS

SEE PROFILE

Anal. Chem. **2009,** *81,* 7484–7489

Technical Notes

Aptamer-Linked Assay for Thrombin Using Gold Nanoparticle Amplification and Inductively Coupled Plasma−Mass Spectrometry Detection

Qiang Zhao, Xiufen Lu, Chun-Gang Yuan, Xing-Fang Li, and X. Chris Le*

Division of Analytical and Environmental Toxicology, Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, AB, Canada T6G 2G3

We describe a sensitive and specific sandwich assay for human α-thrombin. The assay takes advantage of sandwich binding of two affinity aptamers for increased specificity, gold nanoparticles for signal amplification, magnetic beads for fast magnetic separation, and inductively coupled plasma mass spectrometry for ultrasensitive detection. Other proteins, such as immunoglobulin G, serum albumin, transferrin, fibrinogen, and lysozyme did not show interference with the assay for human α-thrombin. The detection limit of human α-thrombin was as low as 0.5 fmol, corresponding to 10 pM thrombin in 50 μL, and the dynamic range covered ~3 orders of magnitude.

Derived from random single-stranded nucleic acid sequence pools and selected by a process called "systematic evolution of ligands by exponential enrichment",[1−5] aptamers rival antibodies as affinity ligands. Once the sequence is known, aptamers are easy to produce with good reproducibility between batches, they are more stable than antibodies, and they can be readily modified (e.g., chemically labeled or immobilized).[6,7] Thus, many recently developed assays and biosensors have shown great promise of aptamers in analytical applications.[8−25] Taking advantage of the recent advances in bioanalytical chemistry and nanotechnology,[8−28] we show here an analytical strategy that achieves both high specificity (using two affinity aptamers to sandwich the target analyte molecule) and the enhanced sensitivity (using affinity gold nanoparticles (Au NPs) for signal amplification). This extends the benefits of successful element-tagged and nanoparticle-labeled immunoassays,[26−41] in which inductively coupled plasma−mass spectrometry (ICP-MS) has been applied as a powerful tool for

* Author to whom correspondence should be addressed. Tel.: (780) 492-6416. Fax: (780) 492-7800. E-mail: xc.le@ualberta.ca.

(1) Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818–822.
(2) Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505–510.
(3) Klussmann, S. Ed. *The Aptamer Handbook, Functional Oligonucleotides and Their Applications*; Wiley−VCH: Weinheim, Germany, 2006.
(4) Stoltenburg, R.; Reinemann, C.; Strehiltz, B. *Biomol. Eng.* **2007**, *24*, 381–403.
(5) Gopinath, S. C. B. *Anal. Bioanal. Chem.* **2007**, *387*, 171–182.
(6) Jayasena, S. D. *Clin. Chem.* **1999**, *45*, 1628–1650.
(7) Bunka, D. H. J.; Stockley, P. G. *Nat. Rev. Microbiol.* **2006**, *4*, 588–596.
(8) Tombelli, S.; Minunni, M.; Mascini, M. *Biosens. Bioelectron.* **2005**, *20*, 2424–2434.
(9) Hamula, C. L. A.; Guthrie, J. W.; Zhang, H.; Li, X.-F.; Le, X. C. *TrAC: Trends Anal. Chem.* **2006**, *25*, 681–691.
(10) Mairal, T.; Özalp, V. C.; Sánchez, P. L.; Mir, M.; Katakis, I.; O'Sullivan, C. K. *Anal. Bioanal. Chem.* **2008**, *390*, 989–1007.
(11) Willner, I.; Zayats, M. *Angew. Chem., Int. Ed.* **2007**, *46*, 6408–6418.
(12) Song, S.; Wang, L.; Li, J.; Zhao, J.; Fan, C. *TrAC: Trends Anal. Chem.* **2008**, *27*, 108–117.
(13) Tang, Z.; Mallikaratchy, P.; Yang, R.; Kim, Y.; Zhu, Z.; Wang, H.; Tan, W. *J. Am. Chem. Soc.* **2008**, *130*, 11268–11269.
(14) Medley, C. D.; Smith, J. E.; Tang, Z.; Wu, Y.; Bamrungsap, S.; Tan, W. *Anal. Chem.* **2008**, *80*, 1067–1072.
(15) He, P.; Shen, L.; Cao, Y.; Li, D. *Anal. Chem.* **2007**, *79*, 8024–8029.
(16) Fischer, N. O.; Tarasow, T. M.; Tok, J. B.-H. *Anal. Biochem.* **2008**, *373*, 121–128.
(17) Xiang, Y.; Xie, M.; Bash, R.; Chen, J. J. L.; Wang, J. *Angew. Chem., Int. Ed.* **2007**, *46*, 9054–9056.
(18) Li, B.; Wang, Y.; Wei, H.; Dong, S. *Biosens. Bioelectron.* **2008**, *23*, 965–970.
(19) Zhou, L.; Ou, L.-J.; Chu, X.; Shen, G.-L.; Yu, R.-Q. *Anal. Chem.* **2007**, *79*, 7492–7500.
(20) Centi, S.; Tombelli, S.; Minunni, M.; Mascini, M. *Anal. Chem.* **2007**, *79*, 1466–1473.
(21) Li, Y.; Zhang, C.; Li, B.-S.; Zhao, L.-F.; Li, X.-B.; Yang, W.; Xu, S.-Q. *Clin. Chem.* **2007**, *53*, 1061–1066.
(22) Zheng, J.; Feng, W.; Lin, L.; Zhang, F.; Cheng, G.; He, P.; Fang, Y. *Biosens. Bioelectron.* **2007**, *23*, 341–347.
(23) Numnuam, A.; Chumbimuni-Torres, K. Y.; Xiang, Y.; Bash, R.; Thavarungkul, P.; Kanatharana, P.; Pretsch, E.; Wang, J.; Bakker, E. *Anal. Chem.* **2008**, *80*, 707–712.
(24) Li, Y.; Lee, H. J.; Corn, R. M. *Anal. Chem.* **2007**, *79*, 1082–1088.
(25) Polksy, R.; Gill, R.; Kaganovsky, L.; Willner, I. *Anal. Chem.* **2006**, *78*, 2268–2271.
(26) Rosi, N. L.; Mirkin, C. A. *Chem. Rev.* **2005**, *105*, 1547–1562.
(27) Wang, J. *Small* **2005**, *1*, 1036–1043.
(28) Katz, E.; Willner, I. *Angew. Chem., Int. Ed.* **2004**, *43*, 6042–6108.
(29) Zhang, H.; Zhao, Q.; Li, X.-F.; Le, X. C. *Analyst* **2007**, *132*, 724–737.
(30) Zhang, C.; Zhang, Z.; Yu, B.; Shi, J.; Zhang, X. *Anal. Chem.* **2002**, *74*, 96–99.
(31) Bandura, D. R.; Tanner, S. D. *Anal. Chem.* **2002**, *74*, 1629–1636.
(32) Quinn, Z.; Baranov, V. I.; Tanner, S. D.; Wrana, J. L. *J. Anal. At. Spectrom.* **2002**, *17*, 892–896.
(33) Zhang, C.; Wu, F.; Zhang, X. *J. Anal. At. Spectrom.* **2002**, *17*, 1304–1307.
(34) Ornatsky, O.; Baranov, V. I.; Bandura, D. R.; Tanner, S. D.; Dick, J. *J. Immunol. Methods* **2006**, *308*, 68–76.
(35) Zhang, S.; Zhang, C.; Xing, Z.; Zhang, X. *Clin. Chem.* **2007**, *50*, 1214–1221.
(36) Merkoci, A.; Aldavert, M.; Tarrasón, G.; Eritja, R.; Alegret, S. *Anal. Chem.* **2005**, *77*, 6500–6503.
(37) Bettmer, J.; Jakubowski, N.; Prange, A. *Anal. Bioanal. Chem.* **2006**, *386*, 7–11.
(38) Hutchinson, R. W.; Ma, R.; McLeod, C. W.; Milford-Ward, A.; Lee, D. *Can. J. Anal. Sci. Spectrosc.* **2004**, *49*, 429–435.

10.1021/ac900961y CCC: $40.75
Published on Web 08/11/2009

trace analysis of elements. The ICP-MS-linked bioassays[30−41] that use elemental tags offer large dynamic range (several orders of magnitude), high sensitivity, high precision, and low detection limits. Au NPs can be directly atomized and measured via ICP-MS,[30−41] avoiding the use of toxic and harmful solution (acidic bromine−bromide solution) to dissolve Au NPs into ions. In addition, the use of magnetic beads as carriers of affinity ligands (antibodies or aptamers) in bioassays has shown advantages over microplates and titers by allowing the binding of targets to the affinity probes in the homogeneous solution, facilitating the kinetics of the binding process, reducing the incubation time, and bringing a fast and effective magnetic separation.[26−29]

To demonstrate the proof of principle, we chose human α-thrombin as the target analyte of interest. Thrombin is a common protein that catalyzes many coagulation-related reactions responsible for blood clotting. Two aptamers have been identified to bind with human α-thrombin: Apt29 (a 29-mer), which binds to the heparin-binding site of thrombin ($K_d \approx 0.5$ nM), and Apt15 (a 15-mer), which binds to the fibrinogen-binding site of thrombin ($K_d \approx 100$ nM).[42,43] Figure 1 schematically shows the principle of the aptamer sandwich assay that is generally applicable to the analysis of macromolecules. To assay for human α-thrombin, the first aptamer (Apt29) is conjugated to gold nanoparticles (Au NPs, 10 nm in diameter). The second aptamer (Apt15) is attached to streptavidin-coated magnetic beads (1 μm in diameter). The binding of the two aptamers to the same thrombin molecule results in a sandwich complex (Apt29−thrombin−Apt15) (see Figure 1). The sandwich complex is then quantified by the determination of gold using ICP-MS. The use of magnetic beads facilitates the capturing of targets and enables a fast magnetic separation of the complex from the unbound reagents. The use of Au NPs brings the signal enhancement for the subsequent detection of gold by ICP-MS. Because ICP-MS measures ^{197}Au elemental ions produced by Au atoms, and because a 10-nm-diameter Au NP contains ~30 000 Au atoms,[34,44] the use of 10-nm Au NPs could potentially increase the sensitivity of the assay up to 30 000-fold.

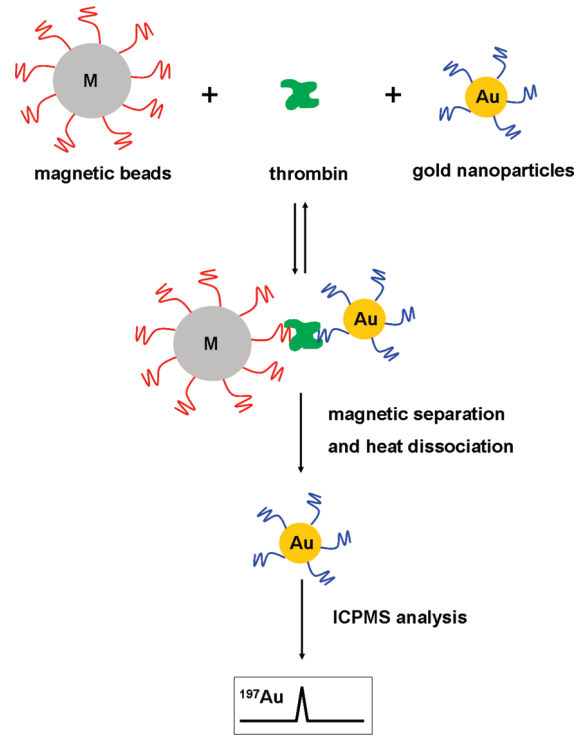

Figure 1. Schematic diagram showing the principle of aptamer-linked sandwich assay for human α-thrombin using gold nanoparticles (Au NPs), magnetic beads, and inductively coupled plasma−mass spectrometry (ICP-MS). Thrombin was sandwiched between two aptamers: the first aptamer (shown in red), which was attached to magnetic beads (identified as M), and the second aptamer (shown in blue), which was conjugated to Au NPs (identified as Au). The sandwich complex was separated from the sample mixture under a magnetic field. After washing, the sandwich complex was heat dissociated (at 90 °C). The magnetic beads were removed and the Au NPs in the solution were analyzed by ICP-MS. The intensity of the ICP-MS measurement of Au at *m*/*z* 197 corresponded to the original concentration of human α-thrombin in the sample.

EXPERIMENTAL SECTIONS

Chemicals and Materials. Solution of gold nanoparticles (Au NPs) (10 nm in diameter, 5.4×10^{12} Au NPs/mL) was obtained from Sigma (Product No. G1527). Immunoglobulin G (IgG), transferrin, human serum albumin (HSA), bovine serum albumin (BSA), human serum (Product No. P2918), and tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were also obtained from Sigma. The aptamers of human α-thrombin used in this study had the following sequences: thiolated 29-mer aptamer (Apt29), 5′-HS-$(CH_2)_6$-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′; biotinylated 15-mer aptamer (Apt15), 5′-biotin-TEG linker-GGT TGG TGT GGT TGG-3′ (TEG: tetraethylene glycol). The thiolated and biotinylated aptamers were synthesized and purified by Integrated DNA Technologies, Inc. (IDT, Coralville, IA). The thiolated aptamers were received in a disulfide form. The polyA_{29} with the modification of biotin and TEG at 5′-end was also obtained from IDT. Streptavidin-coated magnetic beads (1.05 μm, Dynabeads MyOne Streptavidin C1) and a magnetic separator (DYNAL MPC-S) were purchased from Invitrogen Dynal (AS, Oslo, Norway). Human α-thrombin, human β-thrombin, human γ-thrombin, human prothrombin, human antithrombin III, and human fibrinogen were purchased from Haematologic Technologies, Inc. (Essex Junction, VT). A spin cartridge to remove seven high-abundance proteins (serum albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, and fibrinogen) from human serum was obtained from Agilent Technologies, Inc. (Product No. 5188-5341). Solvents and other reagents were supplied by Sigma (Oakville, ON, Canada) and Fisher Scientific (Nepean, ON, Canada). The following buffer solutions were used: buffer A (50 mM Tris-HCl + 2 M NaCl + 0.1% Tween 20, pH 7.4), and buffer B (20 mM Tris-HCl + 140 mM NaCl + 5 mM KCl + 1 mM $MgCl_2$ + 1 mM $CaCl_2$, pH 7.4).

Preparation of Aptamer-Modified Gold Nanoparticles. The thiolated aptamer was conjugated to Au NPs using a modified

(39) Hutchinson, R. W.; Cox, A. G.; McLeod, C. W.; Marshall, P. S.; Harper, A.; Dawson, E. L.; Howlett, D. R. *Anal. Biochem.* **2005**, *346*, 225–233.
(40) Lou, X.; Zhang, G.; Herrera, I.; Kinach, R.; Ornatsky, O.; Baranov, V. I.; Nitz, M.; Winnik, M. A. *Angew. Chem., Int. Ed.* **2007**, *46*, 6111–6114.
(41) Ornatsky, O. I.; Kinach, R.; Bandura, D. R.; Lou, X.; Tanner, S. D.; Baranov, V. I.; Nitz, M.; Winnik, M. A. *J. Anal. At. Spectrom.* **2008**, *23*, 463–469.
(42) Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J. *Nature* **1992**, *355*, 564–566.
(43) Tasset, D. M.; Kubik, M. F.; Steiner, W. *J. Mol. Biol.* **1997**, *272*, 688–698.
(44) Dequaire, M.; Degrand, C.; Limoges, B. *Anal. Chem.* **2000**, *72*, 5521–5528.

literature procedure.[45] Briefly, 50 μL of thiolated Apt29 (70 μM) with the disulfide form was added to 50 μL of 5 mM TCEP and 100 mM Tris-HCl (pH 7.4). The mixture was incubated for 1 h at room temperature to produce the free thiol groups. This solution was then added to 3 mL of the Au NPs solution, and the mixture was maintained at 4 °C for 16 h. To this mixture was added 160 μL of 2 M NaCl. The solution was stored at 4 °C for 24 h. It was centrifuged at 17 000 *g* for 35 min to separate the Au NPs from the unreacted reagents. The Au NPs were washed twice with 10 mM Tris-HCl (pH 7.4), and then the Au NPs were redispersed in 1 mL of 10 mM Tris-HCl (pH 7.4). The solution was diluted 10 times with 10 mM Tris-HCl. The final concentration of the Au NPs solution was ~2 nM, estimated from the absorbance measured at 520 nm. (According to the supplier, the molar extinction coefficient of the Au NPs at 520 nm was 8.8×10^7 M^{-1} cm^{-1}.) The Apt29-modified Au NPs solution was stored at 4 °C when not in use and was determined to be stable for more than two months. Based on the changes in absorbance at 260 nm, it was estimated that ~100 aptamers were modified on each Au NP.

Preparation of Aptamer-Modified Magnetic Beads. The biotinylated aptamer (Apt15) was conjugated to the magnetic beads following the procedure provided by Invitrogen Dynal. Briefly, 4 mg of streptavidin-coated magnetic beads (10 mg/mL) were placed into a 600-μL centrifuge tube and rinsed once with 400 μL of buffer A before use, and then the beads were resuspended in 200 μL of buffer A. Then, 50 μL of biotinylated Apt15 (19 μM) and 150 μL of H_2O were added and the mixture incubated for 2 h under gentle shaking. The magnetic beads were separated from the unreacted reagents on a magnetic separator followed by rinsing with buffer B + 0.1% Tween 20 three times. The magnetic beads were resuspended in 400 μL of buffer B and stored at 4 °C when not in use. It was estimated that, on average, $\sim 1.4 \times 10^5$ aptamers were attached to each magnetic bead.

Formation and Separation of Aptamer-Thrombin Sandwich Complex. The Apt29-modified Au NPs solution was equilibrated with buffer B and 0.1% BSA (1 mg/mL) for 1 h. Five microliters of solution sample or human α-thrombin standard, 43 μL of the Apt29-modified Au NPs (~1 nM), and 2 μL of Apt15 modified magnetic beads suspension were pipetted into a 0.6-mL centrifuge tube, and the mixture was incubated on a shaker at room temperature for 1 h. The tube was placed on a magnetic separator to extract the magnetic beads, which were then rinsed 3 times with 100 μL of buffer B + 0.1% Tween 20. The magnetic beads were redispersed in 100 μL of buffer B + 0.1% Tween 20. The suspension of the magnetic beads was heated at 90 °C for 20 min to dissociate the sandwich complex. The tube was placed on the magnetic separator again. The magnetic beads were pulled off from the solution and were discarded. The solution containing the Au NPs that were released from the sandwich complex was retained for the subsequent analysis of Au by ICP-MS. This solution was diluted 20 times with the solution of 1% HNO_3 + 1% BSA before ICP-MS analysis. For each sample and calibration solution, triplicate incubations were conducted, and six replicate ICP-MS analyses were performed on each replicate of the incubation solutions.

Detection Limit. Detection limit, which is based on 3 times the signal-to-background ratio, was determined from replicate analyses of blanks and α-thrombin calibration solutions. α-Thrombin blank solutions consisted of 5-μL buffer solution, 43 μL of the Apt29-modified Au NPs (~1 nM), and 2 μL of Apt15-modified magnetic beads suspension. The 50-μL mixture was analyzed using the same procedure for human-α-thrombin standard, as described previously. Triplicate preparations of blank solutions were analyzed, and a standard deviation was obtained from all 18 analyses (six ICP-MS analyses of each triplicate blank preparation). Using the calibration curve of α-thrombin, the detection limit was calculated from the signal that was equivalent to 3 times the standard deviation of the blanks.

Specificity Test. To test the specificity of the assay for α-thrombin, nine other proteins were analyzed separately, using the same procedures as those used for human α-thrombin. The concentrations of the test proteins were 10 nM human serum albumin (HSA), immunoglobulin G (IgG), transferrin, lysozyme, fibrinogen, antithrombin III, prothrombin, and human γ-thrombin. The concentration of human β-thrombin was 1 nM. A 5-μL solution that contained a test protein was incubated with 43 μL of a solution of Apt29-modified Au NPs and 2 μL of a suspension of Apt15-modified magnetic beads. The incubation solution was analyzed by ICP-MS, and the results were compared with those obtained from the analyses of 1 nM human α-thrombin. Specificity information was also obtained from the measurements of human α-thrombin spiked into a serum sample matrix (see the subsection entitled "Recovery Test").

Recovery Test. Recovery tests were performed by measuring human α-thrombin added into a human serum sample (from Sigma), either with or without any pretreatment. In the case of sample pretreatment, the serum sample was either diluted 10−20 fold with buffer B and 0.1% BSA (1 mg/mL) or passed through a spin cartridge (Agilent Technologies) to remove seven abundant proteins (serum albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, and fibrinogen). To the untreated, diluted, and the spin-cartridge-treated serum samples was added 5−25 fmol of human α-thrombin. Five-microliter samples that contained the spiked human α-thrombin was subsequently incubated with 43 μL of a solution of Apt29-modified Au NPs (1 nM) and 2 μL of a suspension of Apt15-modified magnetic beads. The incubation solution was analyzed by ICP-MS, and recoveries were obtained from comparing the measured amounts with the added amounts of human α-thrombin. The mean and standard deviation values for recovery were obtained from six replicate ICP-MS analyses of each of the triplicate spike samples.

ICP-MS Analysis. An Agilent 7500cs octopole reaction system (for ICP-MS analysis), operated in the helium mode, was used for the elemental analysis of Au NPs. The operating parameters of ICP-MS are summarized in Table 1. Prior to the analysis, the positions of the torch, the nebulizer gas flow, and the lens were optimized using the tuning solution (1 μg/L each of Li, Mg, Y, Ce, Tl, Co in 2% nitric acid, obtained from Agilent).

A 20-μL sample was injected using a Rheodyne six-port injector (Model 7725i, Rheodyne, Rohnert Park, CA) to a 1% HNO_3 carrier stream. A peristaltic pump (Mandel Scientific, Ontario, Canada) operated at a flow rate of 1.8 mL/min, was used to deliver the carrier and the sample solution. A Teflon tubing (0.3 mm i.d.,

(45) Hill, H. D.; Mirkin, C. A. *Nat. Protocols* **2006**, *1*, 324–336.

Table 1. Operating Parameters of ICP-MS

parameter	value
RF power	1550 W
argon coolant gas	15 L/min
argon carrier gas	0.98 L/min
argon makeup gas	0.25 L/min
helium gas	3.5 mL/min
m/z	197
integration time per point	0.1 s

Supelco) was used to connect the sample injector to the ICP-MS system.

RESULTS AND DISSCUSION

To test whether ICP-MS could quantitatively measure Au NPs, we constructed a calibration curve by measuring the signal intensity of ^{197}Au from the analysis of 20-μL solutions that contained varying amounts of Au NPs (from 1 fM to 10 pM). We used 1% bovine serum albumin (BSA) and 1% HNO_3 to assist the dispersion of Au NPs in solution. The use of BSA and HNO_3 also reduced adsorption of Au NPs on the surfaces of containers and tubing and thus improved the reproducibility of analysis.[31] Six replicate analyses of 50 fM Au NPs gave a relative standard deviation of <2%. The detection of Au NPs by ICP-MS yielded a linear response (R = 0.999) between the signal intensity and the concentration of Au NPs for a dynamic range of 4 orders of magnitude (0.001−10 pM) (see Figure S1 in the Supporting Information).

Having established that ICP-MS was suitable for measuring Au NPs, we further conducted sandwich assays for human α-thrombin. Human α-thrombin was sandwiched by the Apt15-modified magnetic beads and the Apt29-modified Au NPs (see Figure 1). The use of aptamer-modified magnetic beads facilitated a simple magnetic separation of the sandwich complex from the sample mixture and the excess reagents. The aptamer-modified Au NPs served as affinity probes for ultrasensitive detection of thrombin, using ICP-MS. The quantitative measure of ^{197}Au was directly proportional to the amount of thrombin that was sandwiched by the two aptamers in the original sample.

Figure 2 shows typical results obtained from six replicate analyses of human α-thrombin (0, 0.05, 0.2, and 1 nM). Additional detailed analyses of thrombin in the concentration range from 0.01 nM to 10 nM showed a good linear relationship (R = 0.997) between the signal intensity and the thrombin concentration in the range of 0.05−1 nM (see Figure 3). The linear dynamic range could be further extended at the higher concentration end (>5 nM) by increasing the concentration of the aptamer-functionalized reagents. The detection limit, which is defined as 3 times the standard deviation of the background, was 0.01 nM human α-thrombin in the 50-μL reaction mixture or 0.1 nM human α-thrombin in the 5-μL original sample solution, corresponding to 0.5 fmol of human α-thrombin. This detection limit is better than or comparable to those reported by most of aptamer-based assays for thrombin,[8−12,17,20,22−25,29] as summarized in Table S1 in the Supporting Information. Six replicate ICP-MS analyses showed a relative standard deviation (RSD) of <6%. Triplicate analyses of solutions that contained 0.05, 0.2, and 1 nM thrombin exhibited RSDs of <11%.

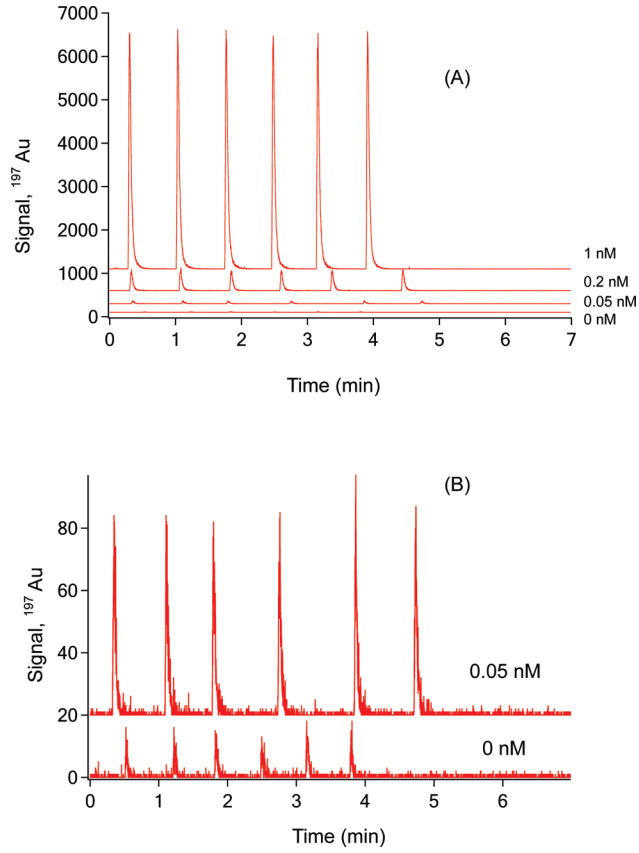

Figure 2. Typical analytical signals showing replicate analyses of human α-thrombin (from bottom to top curves: 0, 0.05, 0.2, and 1 nM) using the aptamer sandwich assay. (B) The graph represents the expanded traces from the analyses of 0.05 nM human α-thrombin and reagent blank. The reproducible signals were from six repeat ICP-MS analyses of gold that was originated from the human α-thrombin sandwiched by two aptamers (one conjugated to gold nanoparticles (Au NPs) and the other to magnetic beads). The intensity of ^{197}Au signal corresponded to the human α-thrombin concentration in the 50-μL original reaction mixture.

The background signals observed from the reagent blank (Figure 2B) could be due to interactions between the two aptamers (Apt15 and Apt29). High background signals have been reported in the literature,[15] but no reason was given. We hypothesize that these two aptamers could form intermolecular G-quartet structures,[42,43,46] because of their G-rich sequences. We tested this hypothesis by immobilizing a poly A sequence (polyA$_{29}$) on the magnetic beads instead of the Apt15 aptamer, following the same experimental procedures. PolyA$_{29}$ could not interact with Apt29 on the Au NPs to form the G-quartet structure. In the control experiments in which no target human α-thrombin was added, the polyA$_{29}$-modified magnetic beads were tested along with the Apt29-modified AuNPs, followed by incubation, washing and separation, heat release, and ICP-MS analysis. We observed a reduced background signal of Au NPs (about 4-fold decrease); this is consistent with the hypothesis. Reducing the amounts of the two aptamers in the assay would reduce the background. However, an aptamer concentration that is too low would lead to inefficient binding to the thrombin targets. Therefore, the amounts of the aptamer-modified magnetic beads and Au NPs used in the assay were optimized for the

(46) Davis, J. T. *Angew. Chem., Int. Ed.* **2004**, *43*, 668–698.

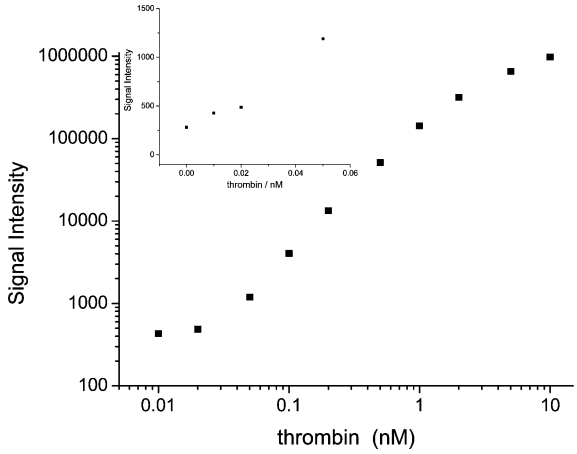

Figure 3. Detection of human α-thrombin in standard solutions (0.01−10 nM) using the aptamer sandwich assay. Within a concentration range of 0.05−1 nM, linear correlation (R = 0.997) was observed between the signal intensity of ICP-MS and the concentration of human α-thrombin (0.05−1 nM) in the 50-μL original reaction mixture. The results were average from triplicate analyses. The inset shows the results obtained from lower concentrations of thrombin (0−0.05 nM). The RSDs were (at the indicated concentration): 4%, (0 nM), 5% (0.01 nM), 6% (0.02 nM), 5% (0.05 nM), 4% (0.1 nM), 1% (0.2 nM), 1% (0.5 nM), 1% (1 nM), 1% (2 nM), 3% (5 nM), and 2% (10 nM).

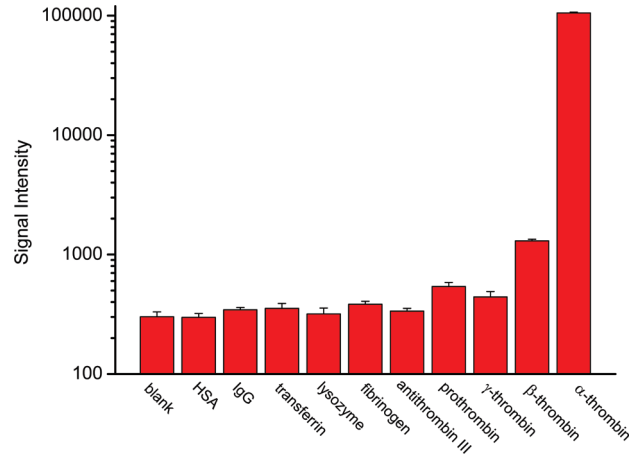

Figure 4. Testing of human α-thrombin and other proteins, using the aptamer sandwich assay, showing the specificity of the assay for human α-thrombin (1 nM) in the 50-μL original reaction mixture. The concentration of other proteins were 10 nM for HSA, IgG, transferrin, lysozyme, fibrinogen, human antithrombin III, human prothrombin, and human γ-thrombin, and 1 nM human β-thrombin in the 50-μL original reaction mixture.

best signal-to-background ratio. In addition, the same batches of the prepared aptamer-modified magnetic beads and Au NPs were used in the assay to control the reproducibility.

We further tested the specificity of the aptamer-based sandwich assay for human α-thrombin. We tested nine proteins in place of human α-thrombin, using the same experimental procedures as those for human α-thrombin. We selected these proteins for testing because they are either abundant proteins in serum (e.g., HSA, IgG, fibrinogen, and transferrin) or, similar to α-thrombin, they participate in blood coagulation (e.g., fibrinogen, antithrombin III, prothrombin, β-thrombin, and γ-thrombin). We also tested lysozyme, which is a positively charged protein at neutral pH, as a worst-case scenario for its potential nonspecific interaction with the negatively charged DNA. Figure 4 shows that 10-fold excess of HSA, IgG, transferrin, lysozyme, fibrinogen, antithrombin III, and prothrombin did not interfere with the assay for a trace level (1 nM) of human α-thrombin. Human β-thrombin and human γ-thrombin share some similarity in structure with human α-thrombin, and they are the proteolyzed products of human α-thrombin, through enzymatic cleavage or autolytic cleavage.[47] These two proteins were also tested by following the same experimental procedures. A 10-fold excess of human γ-thrombin did not produce an interfering signal. Human β-thrombin was the only protein tested that could be detected along with human α-thrombin. However, the signal obtained from β-thrombin was ~100-fold lower than that produced from α-thrombin at the same concentration. Furthermore, the concentrations of β-thrombin in human serum samples are expected to be much lower than α-thrombin; the reported β-thrombin levels were ~2.6% ± 3.1% of total thrombin.[48] Therefore, the levels of β-thrombin are not significant in causing interference with the determination of α-thrombin in human serum samples. These results indicate that the aptamer-based sandwich assay for human α-thrombin exhibits very good specificity. Furthermore, the presence of 15 000-fold excess BSA (0.015 mM) in the assay buffer did not affect the assay for human α-thrombin.

Table 2. Recovery of Human α-Thrombin Spiked into Human Serum Samples

sample treatment	spiked thrombin	recovery (%)
serum, no treatment	5−25 fmol	not detectable
cartridge, no dilution	10 fmol	8 ± 4
cartridge, 5-fold dilution	5 fmol	62 ± 7
serum, 10-fold dilution	5−25 fmol	93 ± 7
serum, 20-fold dilution	25 fmol	104 ± 9

We further tested the analyses of human α-thrombin (5−25 fmol) spiked into 5 μL of human serum samples, using the same experimental procedures as those for the human α-thrombin standard. Results in Table 2 show that the untreated serum samples pose severe interference with the analysis of human α-thrombin. After removing seven abundant proteins (serum albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, and fibrinogen) from human serum samples, using an affinity spin cartridge, the recovery of human α-thrombin spiked to the undiluted serum was 8%. A 5-fold dilution of the serum samples, combined with the removal of abundant proteins prior to analysis, resulted in an improvement in recovery to 62%. Acceptable recoveries (93% ± 7%) were obtained from the analyses of thrombin spiked in 10-fold-diluted serum samples, with or without the removal of abundant proteins. Recoveries of thrombin from 20-fold-diluted serum samples were 104 ± 9%. It appears that diluted serum samples (by 10-fold or more) have little matrix effects on the analysis of thrombin. Dilution of serum samples by 10-fold prior to analysis is a common practice for the determination of thrombin using aptamers or antibodies as affinity probes.[16,20]

Comparing the results shown in Figure 3 and Figure S1 in the Supporting Information, there appears to be a 1000-fold

(47) Davie, E. W.; Kulman, J. D. *Semin. Thromb. Hemostasis* **2006**, *32* (1), 3–15.

(48) Fenton, J. W.; Fasco, M. J.; Stackrow, A. B.; Aronson, D. L.; Yong, A. M.; Finlayson, J. S. *J. Biol. Chem.* **1977**, *252*, 3587–3598.

difference in signal intensity between thrombin (Figure 3) and the Au NPs (Figure S1 in the Supporting Information). This difference is due to two main reasons. First, in the case of thrombin analysis (Figure 3), the Au NPs that were released from magnetic beads were further diluted 20 times with 1% HNO_3 and 1% BSA before the ICP-MS analysis. The signals in Figure 3 were obtained from 20-fold-diluted thrombin-bound nanoparticles. The second reason for lower signal from the thrombin-aptamer nanoparticles than that from the free nanoparticles is that each Au NP is attached to ~100 aptamers. Therefore, each Au NP may bind to more than one target molecule. Although the best efficiency could be expected when only one aptamer is attached to each Au NP, the use of a larger number of aptamers conjugated to the Au NPs helps to improve the efficiency of the aptamer binding to the target.

In conclusion, we have demonstrated a novel sandwich assay for human α-thrombin by taking advantage of two affinity aptamers for the increased specificity, the use of gold nanoparticles (Au NPs) for signal amplification, and inductively coupled plasma mass spectroscopy (ICP-MS) for highly sensitive detection. The use of magnetic beads facilitated the capture of targets in homogeneous solution and provided fast and efficient magnetic separation of the affinity complex. Thus, this assay was accomplished in a shorter time than other sandwich immunoassays using microplates and antibodies.[30] Both aptamers (conjugated magnetic beads and Au NPs) were very stable; they could be stored at 4 °C for a few months without any loss of activity. The use of ICP-MS took advantage of its high sensitivity and wide linear dynamic range. Using Au NPs >10 nm in size, the sensitivity of the present assay could be further improved, because of the greater signal amplification by the larger Au NPs. A caveat of using larger Au NPs may be the possible space hindrance in target binding. However, when detecting targets at extremely low concentrations, the space hindrance is not expected to be a problem, because only small numbers of Au NPs and magnetic beads would be brought together by the minute amounts of the targets. In addition to Au NPs, other nanaoparticles (e.g., quantum dots and silver nanoparticles), can also be used as labels. Because ICP-MS is capable of specific detection of multielements, high-throughput analyses of multiple targets can be achieved by labeling aptamers for different targets with different elemental tags. This assay opens up new opportunities for broad analytical applications of aptamers.

ACKNOWLEDGMENT

The work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC), Canadian Institutes of Health Research, Canada Research Chairs (CRC) Program, and Alberta Health and Wellness.

SUPPORTING INFORMATION AVAILABLE

Determination of Au NPs using ICP-MS coupled with flow injection (Figure S1) and a comparison of aptamer-based assays for human α-thrombin (Table S1). (A list of referenced literature is included.) (PDF) This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review May 4, 2009. Accepted July 25, 2009.

AC900961Y