Ultrasensitive Densitometry Detection of Cytokines with Nanoparticle-Modified Aptamers

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Background: Aptamers mimic properties of antibodies and sometimes turn out to be even better than antibodies as reagents for assays. We describe the establishment of an ultrasensitive densitometry method for cytokine detection by nanoparticle (NP)-modified aptamers.

Methods: The assay simultaneously uses a gold NP-modified aptamer and a biotin-modified aptamer to bind to the target protein, forming a sandwich complex. The absorbance signal generated by the aptamer-protein complex is amplified and detected with a microplate reader.

Results: The assay for platelet-derived growth factor B-chain homodimer (PDGF-BB) was linear from 1 fmol/L to 100 pmol/L ($R^2 = 0.9869$). The analytical detection limit was 83 amol/L. The intraassay and interassay imprecision (coefficient of variations) was $<7.5\%$.

Serum concentrations of PDGF-BB determined with the gold NP-modified aptamer assay and with ELISA were not significantly different.

Conclusions: The gold NP-modified aptamer assay provides a fast, convenient method for cytokine detection and improves the detection range and the detection limit compared with ELISA.

Current methods for the analysis of cytokines and growth factors are mostly immunologic assays, and antibodies are the most commonly used affinity reagents (1–5). Aptamers can rival antibodies as affinity ligands and have been used in numerous studies of the detection of proteins (6). Aptamers are short synthetic nucleotide sequences (DNA or RNA) that have been selected in in vitro selection experiments via the systematic evolution of ligands by exponential enrichment (SELEX)1 process to act as ligands with high-affinity binding to their targets (7). Once the sequences of DNA aptamers have been identified, their straightforward synthesis makes them particularly desirable. Although aptamers are different from antibodies, they mimic the properties of antibodies in a variety of diagnostic formats (6). The demand for diagnostic assays to assist in the management of existing and emerging diseases is increasing, and aptamers have the potential to fulfill the requirement for molecular recognition in such assays (8).

Several aptamer-based methods for protein detection have been developed. Fluorescence anisotropy has been used to measure platelet-derived growth factor (PDGF) with a fluorescein-labeled single-stranded DNA aptamer (9), and molecular aptamer beacons have been designed for the detection of proteins (10–13). Fredriksson et al. (14) and Wang et al. (15) have established highly sensitive detection methods by combining aptamer recognition with the sensitivity of real-time quantitative PCR. Although real-time PCR–based methods can detect zeptomole quantities of protein, real-time PCR instruments are still required. Huang et al. developed a colorimetric assay that uses gold nanoparticles (NPs) to measure PDGFs and their receptors (16). The assay was not useful for clinical monitoring, which requires measurements in the nanomolar range.

In the present study, we developed an ultrasensitive densitometric cytokine-detection method that uses gold NP-modified aptamers. The assay relies on aptamer pairs that bind to 2 distinct sites on the target protein or to the same epitope of a homodimer. Binding of the aptamers to the target protein forms a sandwich complex, which is immobilized onto a microplate via avidin. After silver

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1 Nonstandard abbreviations: SELEX, systematic evolution of ligands by exponential enrichment; PDGF, platelet-derived growth factor; NP, nanoparticle; BB, B-chain homodimer.
enhancement, the signal is amplified, and a microplate reader measures the absorbance.

**Materials and Methods**

**MATERIALS**

Oligonucleotides modified with a thiol group and biotin were synthesized by Invitrogen Biotech and used without further purification (sequence 1, 5′-SH-(CH$_2$)$_5$-AGTTCTT-ACTCAGGGCACTTGGCAAGCAATTTGCTGCTCATTGCCAGTGTAT-3′; sequence 2, 5′-Bio-GCAAGTACTCA-GGGCACTTGGCAAGCAATTTGCTGCTCATTGCCAGTGTAT-3′). The underlined sequences correspond to the aptamer sequences, which Green et al. identified via the SELEX process from a library of 3 × 10$^{14}$ molecules (500 pmol) of single-stranded DNA randomized at 40 contiguous positions (17, 18). PDGF-AA, -AB, and -B-chain homodimer (BB) were purchased from Sigma-Aldrich. The RayBio Human PDGF-BB ELISA Kit was purchased from RayBiotech. Gold NPs, 15 nm in diameter, and streptavidin were provided by SABC. AgNO$_3$, citric acid, trisodium citrate, and 1,7 g/L bovine serum albumin. The solution was transferred into the microplate wells and incubated at 37°C for 30 min. The plates were then washed with phosphate-buffered saline (0.2 mol/L NaCl, 20mM Na$_2$HPO$_4$/NaH$_2$PO$_4$ pH 7.0) for 5 min and twice with a 2X buffer containing 0.3 mol/L NaN$_3$ and 10 mmol/L Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 7.0, for 5 min.

**Preparation of Gold NP–Modified Aptamer and Avidin-Coated Microplates**

Gold NP–modified aptamers were prepared as described by Mirkin et al. (19) and modified by Zhang et al. (20) at the storage step at 4°C. Fifty microliters of Tris-EDTA (pH 7.4, containing 10 mmol/L Tris-HCl and 1 mmol/L EDTA) buffer containing 300 nmol/L thiol-modified aptamer probe (sequence 1) was added to the precipitated gold NPs of 500 μL gold NPs. We stored the mixture at 4°C for 12 h, added another 50 μL Tris-EDTA buffer containing 0.2 mol/L NaCl, and stored the aptamer mixture at 4°C for another 24 h. We then purified the NP-modified aptamer probe by centrifugation at 13 600 g for 10 min at 4°C, washing with ultrapure water, and precipitating the probe twice again to eliminate free oligonucleotides. Finally, the gold NP–modified aptamer probe was dissolved in 100 μL Tris-EDTA buffer containing 0.1 pmol/L NaCl.

The avidin-coated microplates were prepared by diluting avidin in sodium carbonate buffer (0.16 g Na$_2$CO$_3$, 0.29 g NaHCO$_3$, diluted in ultrapure water to a final volume of 100 mL, pH 9.6) to a final concentration of 5 μg/mL, immobilizing the avidin on microplates, and incubating the plates overnight at 4°C.

**Detection of PDGF-BB with NP-Modified Aptamer**

We incubated 20 μL of 10 nmol/L biotin-labeled aptamer probe (sequence 2) and 20 μL of an 8 nmol/L solution of gold NP–modified aptamer probe (sequence 1) at room temperature for 20 min with 10 μL of 10-fold serial dilutions of PDGF-BB (1 amol/L to 10 nmol/L) containing 137 mmol/L NaCl, 10.1 mmol/L Na$_2$HPO$_4$ (pH 7.4), 2.7 mmol/L KCl, 1 mmol/L MgCl$_2$, and 10 g/L bovine serum albumin. The solution was transferred into the microplate wells and incubated at 37°C for 30 min. The plates were then washed with phosphate-buffered saline (0.2 mol/L NaCl, 20mM Na$_2$HPO$_4$/NaH$_2$PO$_4$ pH 7.0) for 5 min and twice with a 2X buffer containing 0.3 mol/L NaN$_3$ and 10 mmol/L Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 7.0, for 5 min.

Signal enhancement was carried out by incubating the wells in 100 μL enhancer solution (0.5 g AgNO$_3$ in 2 mL H$_2$O, 1.7 g hydroquinone in 30 mL H$_2$O, 2.5 g citric acid, and 2.35 g trisodium citrate in 10 mL H$_2$O, all mixed together simultaneously immediately before use) at room temperature (21). The enhancement time was 60–80 s. The reaction was terminated by immersing the microplates in doubly distilled water.

Although most of the enhancer can be used in daylight, covering the microplates or working under subdued light conditions is recommended because high light intensities promote the self-nucleation of silver ions, which may confound the results.

**RESULTS**

**Design Strategy of the NP-Modified Aptamer Assay**

Fig. 1 illustrates the scheme of the NP-modified aptamer assay. It relies on the aptamer pairs binding to 2 distinct sites on the target protein or to the same epitope of a homodimer. The gold NP–modified aptamer and the biotin-labeled aptamer were incubated with the target protein to form a sandwich complex, which was immobilized onto the microplate via avidin. To facilitate the visualization of gold NP signals, we designed a signal-amplification procedure in which silver ions were reduced by hydroquinone to silver metal at the surface of the gold NPs; this deposition of silver on the gold NPs amplifies
the absorbance signal, which is recorded densitometrically (Fig. 1).

QUANTIFICATION OF PDGF-BB WITH THE NP-MODIFIED APTAMER ASSAY

To verify the assay experimentally, we used 10-fold serial dilutions of PDGF-BB (1 amol/L to 10 nmol/L) and an 80-s silver-enhancement time to generate a calibration curve. The absorbance at 630 nm was plotted against known PDGF-BB concentrations (Fig. 2); each point represents the mean of duplicate measurements of 3 separate experiments. The analytical detection limit, calculated as the mean background signal $\pm 2$ SD, was 83 amol/L in the 50-μL reaction system. The absorbance at 630 nm was linearly related to PDGF-BB concentration between 1 fmol/L and 100 pmol/L. The calibration curve was described with the equation:

$$y = kx + b,$$

where $y$ is the absorbance, $x$ is the logarithm of the PDGF-BB concentration, $k$ is the slope, and $b$ is the $y$ intercept. In this study, the equation was:

$$y = 0.1943x + 3.1019,$$

with a correlation coefficient ($r^2$) of 0.9896.

SILVER ENHANCEMENT IN THE NP-MODIFIED APTAMER ASSAY

We used silver-enhancement technology to amplify the signals of gold NPs. An image of a microplate containing different PDGF-BB concentrations in this assay is shown in Fig. 3A. The background gray scale of the silver-enhancer solution increased with time (Fig. 3A). The NPs, which serve as nucleation sites for catalysis of silver-ion reduction (22), significantly accelerate the deposition of silver particles. In other words, the photodensity of NP-based silver enhancement was produced in a concentration- and time-dependent manner. To determine the optimal silver-deposition time, we measured the background absorbance with a microplate reader after varying the silver-deposition time from 0 to 100 s. Blank signals were low for silver-deposition times of 20–80 s (Fig. 3B); however, the background increased rapidly when the silver-deposition time exceeded 80 s. We thus chose a time of 60–80 s for silver enhancement.

REPRODUCIBILITY, RECOVERY, AND SPECIFICITY

We evaluated the method’s intraassay imprecision by consecutively analyzing the same samples 5 times and assessed interassay imprecision by analyzing the same samples on 5 consecutive days. The results are shown in Table 1.

Various known PDGF-BB concentrations were added to 2 biological samples (fetal calf serum and cerebrospinal fluid) and a culture medium (Eagle’s minimal essential medium). Mean (range) recoveries were 98% (86%–115%).

PDGF-AA homodimer and PDGF-AB heterodimer were used to evaluate the specificity of this assay. PDGF-AA and PDGF-AB are 2 other PDGF isoforms. They were each incubated with the PDGF-BB aptamer...
and processed in the same way as PDGF-BB. Neither PDGF-AA nor PDGF-AB was detected in the assay (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue6).

COMPARISON OF THE NP-MODIFIED APTAMER ASSAY AND ELISA

The concentration–response relationships of the NP-modified aptamer assay and ELISA for detecting PDGF-BB are shown in Fig. 4. The detection limit of the NP-modified aptamer assay was 83 amol/L, ~1000-fold lower than that of ELISA. The linear range for the NP-modified aptamer assay extends over a 10^6-fold concentration range (1 fmol/L to 100 pmol/L), whereas the ELISA spans 4 orders of magnitude (10 pmol/L to 10 nmol/L). In addition, the entire NP aptamer assay procedure requires only 1 h, whereas the ELISA requires ~5 h.

DETECTION OF PDGF-BB IN SERUM

We analyzed different serum samples from patients with hepatic cirrhosis with both the NP-modified aptamer assay and ELISA. The measured PDGF-BB concentrations are shown in Table 2. PDGF-BB concentrations in the hepatic cirrhosis patients with and without ascites were significantly higher than in the healthy controls, and PDGF-BB concentrations in the hepatic cirrhosis patients with ascites were much higher than in those without ascites. The results with the NP-modified aptamer assay were consistent with those obtained with ELISA. The coefficient of variations of the NP-modified aptamer assay was 12.5%–24.06%, whereas that of ELISA was 17.29%–34.04%.

**Table 1. Intraassay and interassay imprecision for the NP-modified aptamer assay.**

<table>
<thead>
<tr>
<th>PDGF-BB concentration</th>
<th>Coefficient of variations, %*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraassay</td>
</tr>
<tr>
<td>100 amol/L</td>
<td>4.8</td>
</tr>
<tr>
<td>100 fmol/L</td>
<td>3.5</td>
</tr>
<tr>
<td>100 pmol/L</td>
<td>3.3</td>
</tr>
</tbody>
</table>

*a n = 5.

**Table 2. PDGF-BB concentrations in sera.**

<table>
<thead>
<tr>
<th>Samples</th>
<th>NP-modified aptamer assay, ng/L*</th>
<th>ELISA, ng/L*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>0.0008 (0.0001)</td>
<td>0.001 (0.0002)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Hepatic cirrhosis without ascites</td>
<td>89.7 (13.7)</td>
<td>90.2 (15.6)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Hepatic cirrhosis with ascites</td>
<td>180.4 (43.4)</td>
<td>178.6 (60.8)</td>
<td>&gt;0.1</td>
</tr>
</tbody>
</table>

*a Data are presented as the mean (SD).

**Discussion**

Cytokines, products of cells of the immune system, may stimulate the immune response and cause the regression of some cancers. Cytokine detection and quantification play essential roles in clinical practice, as well as in basic research. Methods currently used for cytokine analysis include ELISA (5), membrane protein arrays (2), immunohistochemistry (3), electrochemiluminescence (23), sandwich immunossays with microspheres (4), and flow cytometry (1). Antibodies are currently the most commonly used affinity reagents for these immunologic assays. Molecular probes based on nucleic acid platforms are emerging as complements to antibody-based affinity ligands. DNA and RNA sequences that recognize specific target analytes are known as aptamers (24, 25). Although aptamers are structurally different from antibodies, they mimic properties of antibodies in a variety of diagnostic formats (6). Metallic NPs (e.g., gold NPs) possess various characteristics (including no radioactivity, high electron density, and high sensitivity) that are extensively exploited in different bioanalytical applications (26–28).

Taking advantage of these properties of aptamers and gold NPs, we developed a novel densitometric assay for cytokines that is based on NP-modified aptamers. The signals of gold NPs are amplified through the use of silver-enhancement technology, which is based on the use of hydroquinone as the reducing agent. Hydroquinone facilitates the colloidal gold–catalyzed reduction of silver ions (from silver lactate or silver acetate) to metallic silver on the particle surface (29). Enhancement occurs via the deposition of silver from the solution onto the surface of gold NPs. Each colloidal gold particle thus acts as a catalytic site, and the shell of metallic silver that forms around the gold particles also catalyzes the reaction, producing multiple layers of silver atoms around the gold NPs. Furthermore, silver deposition is proportional to the concentration of the gold NPs, which in turn is proportional to the concentration of the target protein. The power of the silver-enhancement effect is such that a low degree of binding to NPs becomes detectable, greatly promoting the assay’s sensitivity (29). Use of silver-
enhancement technology enables the detection of concentrations as low as 83 amol/L PDGF-BB, and the detection range of the assay can reach 7 orders of magnitude. The sensitivity of this method is superior to that of the reported aptamer-functionalized gold-NP assay for the optical detection of thrombin with a quartz crystal microbalance (detection limit, 2 nmol/L) (30) and is close to that of 2 other assays that use amplification approaches: multiplexed protein profiling on microarrays by rolling-circle amplification (31) and colorimetric bio-barcode amplification (32).

In addition, the absorbance of NP-based silver enhancement is dose- and time-dependent. Prolonging the silver-deposition time increases the background of the gray scale of the silver-enhancer solution; thus, the silver-deposition time is a critical factor for this method.

This method has a sensitivity comparable to the sensitivities reported for PCR-based methods (14, 15); however, our assay is more convenient and does not require complicated instrumentation. The entire procedure is similar to ELISA, and the prerequisite instrument, a microplate reader, is available in almost all laboratories. Compared with immunologic methods for cytokine detection such as ELISA, our assay with an NP-modified aptamer possesses significantly increased sensitivity. Moreover, it uses aptamers as affinity ligands, which are simpler and more flexible to design, prepare, and apply. Compared with the batch-to-batch variability of antibody production, which relies on animals or cell cultures, aptamers are produced by chemical synthesis with very high accuracy and reproducibility (6). These synthetic oligonucleotides have greater temperature stabilities and longer shelf lives than most antibodies (33). Aptamer isolation can be automated and is readily scaled up (34). Moreover, aptamer probes can be shared within the research community via publication of the nucleotide sequence, thereby facilitating the standardization of protein assays between laboratories.

On the other hand, several limitations also exist. Light is an interference factor in silver enhancement, because high light intensity will promote the self-nucleation of silver ions, which may obscure the results. We therefore recommend that microplates be covered or experiments be carried out under a red safe light. In addition, higher temperatures and movement of the microplates during incubation in the enhancer solution may speed up silver deposition on the gold surface. Another limitation resides in the aptamers themselves. Currently, high-affinity aptamers are not as widely available as antibodies. The SELEX process is evolving, however, and new selection protocols will facilitate more efficient aptamer selection and create a more diverse aptamer pool, both of which will greatly extend the application of this new method. To date, several aptamers that have good affinity and good specificity have been characterized for a number of cytokines, including vascular endothelial growth factor (35), basic fibroblast growth factor (36), 1-selectin (37), interferon-γ (38), and others. We believe this method has a promising future in the field of protein detection.

In conclusion, this approach has attractive performance features, such as simplicity and high sensitivity. The method may contribute to the growing number of applications of nanotechnology and may find a use in diagnostics and therapeutics.

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References


