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PAPER

Aptameric system for the highly selective and ultrasensitive detection of protein in human serum based on non-stripping gold nanoparticles

Yanhua Sun, Sheng Cai, Zhijuan Cao, Chaiwan Lau and Jianzhong Lu*

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A novel approach is proposed in this study for the development of an aptameric assay system for protein based on non-stripping gold nanoparticles (NPs)-triggered chemiluminescence (CL) upon target binding. The strategy chiefly depends on the formation of a sandwich-type immunocomplex among the capture antibody immobilized on the polystyrene microwells, target protein and aptamer-functionalized gold NPs. Introduction of target protein into the assay system leads to the attachment of gold NPs onto the surface of the microwells and thus the assembled gold NPs could trigger the reaction between luminol and AgNO_3 with a CL emission. Further signal amplification was achieved by a simple gold metal catalytic deposition onto the gold NPs. Such an amplified CL transduction allowed for the detection of model target IgE down to the 50 fM, which is better than most existing aptameric methods for IgE detection. This new protocol also provided a good capability in discriminating IgE from nontarget proteins such as IgG, IgA, IgM and interferon. The practical application of the proposed gold NPs-based immunoassay was successfully carried out for the determination of IgE in 35 human serum samples. Overall, the proposed assay system exhibits excellent analytical characteristics (*e.g.*, a detection limit on the attomolar scale and a linear dynamic range of 4 orders of magnitude), and it is also straightforward to adapt this strategy to detect a spectrum of other proteins by using different aptamers. This new CL strategy might create a novel technology for developing simple biosensors in the sensitive and selective detection of target protein in a variety of clinical, environmental and biodefense applications.

Introduction

Comparable to antibodies but much easier to obtain, DNA aptamers can be selected for any targets.^{1–7} While antibody-based immunoassay methodologies constitute routine tools for protein detection, aptamers as attractive alternatives to antibodies have become molecular recognition elements in biosensor applications due to the enormous versatility of nucleic acid components in labeling, immobilization, signaling, and amplification. On the basis of aptamers, many aptasensors for proteins have been developed with mechanical, optical, or electronic readouts because the detection and quantification of proteins play essential roles in fundamental research and clinical applications.^{2,8–12} However, very few aptasensors have been successfully employed for the detection of target molecules in human serum. Accurate detection of diagnostic proteins in human serum is often challenging because of its complex composition comprising thousands of proteins with concentrations ranging over 12 orders of magnitude. Albumin, for example, constitutes approximately

half of the total serum protein (30–50 mg mL⁻¹), while many important disease-related biomarkers exist at concentrations as low as 1 pg mL⁻¹.^{13–15}

In recent years, a new aptasensor for the detection of protein using metal nanoparticles (NPs) has emerged.^{16–19} Metal NPs offer excellent prospects for chemical and biological sensing due to their unique optical and mechanical properties.^{20–23} Colloidal Au NPs, which can be readily prepared in a wide range of sizes (from about 2 nm to above 100 nm), as an amplification tag, have been the subject of research directed at gene analysis and antibody or antigen detection. The sensitivity of smaller Au NPs is usually enhanced by silver staining²⁴ and their detecting concentration after silver staining can often reach a level of approximately ng mL⁻¹.^{25–27} This is still much lower than that of fluorescent, radioactive, and enzyme-colorimetric methods.^{28–30} Another alternative has been reported by Ma *et al.*³¹ in which a mixture of HAuCl_4 and reductants was utilized to enlarge the immune Au NPs immobilized on a nitrocellulose strip, and this method offered better sensitivity for detection of immunoglobulin G in comparison with the silver enhancement method. Growth of aptamer-functionalized Au NPs for optical detection of thrombin on a solid substrate is another illustration representing an amplification path by using a mixture of HAuCl_4 ,

School of Pharmacy, Key Laboratory of Smart Drug Delivery, Ministry of Education & PLA, Fudan University, 826 Zhangheng Road, Shanghai, 201203, China. E-mail: jzlu@fudan.edu.cn; Fax: (+86)21-51980058; Tel: (+86)21-51980058

NADH and CTAB.³² In addition, Cao *et al.*³³ introduced another homogenous detection assay that incorporated the Au nanogrowth in a homogenous phase containing HAuCl₄, CTAB and ascorbic acid with the use of magnetic beads to quantify prostate specific antigen based on sandwich immune reactions. Herein we use a mixture of HAuCl₄ and NH₂OH, instead of silver staining solution, to enlarge Au NPs immobilized on the 96-well plate, based on the seeding method of detecting immunoglobulin E (IgE).

Chemiluminescence (CL) has been exploited for a wide range of applications in various fields owing to its extremely high sensitivity, simple instrumentation, wide calibration ranges and suitability for miniaturization in analytical chemistry. Recently, we reported a novel, sensitive CL immunoassay based on a colloidal gold label that, after oxidative gold metal dissolution in an acidic solution, was indirectly quantified by a simple and sensitive luminol CL reaction.²⁶ Our group further developed a sensitive Au NPs-based CL detection assay for the analysis of DNA hybridization.²⁷ The proposed CL protocol was evaluated for a 30-base model DNA sequence, and the detection limit was found to be 0.01 pmol. Further signal amplification was achieved by the assembly of biotinylated Au NPs onto the surface of streptavidin-coated polystyrene beads. Such an amplified CL transduction allowed for the detection of DNA targets down to the 100 amol level. However, the dissolution of Au NPs needed to be conducted under extremely strict conditions (0.01 M HCl–0.5 M NaCl–0.5 mM Br₂). Liu *et al.*²⁸ subsequently found that Ag NP probes could be easily dissolved in a dilute HNO₃ solution and subsequently developed an Ag NPs-based CL method for the ultrasensitive detection of DNA hybridization. However, dissolved Ag⁺ ions needed to be determined by a complicated K₂S₂O₈–Mn²⁺–H₃PO₄ reaction in a 90 °C water bath for 7 min. In addition, Ding *et al.*²⁹ reported flow injection CL detection of DNA hybridization using nanoCuS tags, where a preconcentration process of cupric ions was performed by ASV technology to improve its sensitivity. All of these detection methods are sensitive, however they are comparatively time-consuming and complicated, and often require specialized and cumbersome technical processing, we refer the reader to the recent review by Fan *et al.* in 2009.³⁰ Thus an ultrasensitive, simple and user-friendly technique for the detection of DNA hybridization is in urgent need.

IgE plays a key role in allergic responses. It is the immunoglobulin component found in the lowest levels in human serum. IgE levels, however, increase in patients afflicted with allergic asthma, atopic dermatitis, and other immune deficiency related diseases, including AIDS.^{34–36} There are several reports on successful DNA aptamer-based sensors for IgE (Table 1). For example, Liss *et al.*³⁷ developed a quartz crystal microbalance (QCM)-based aptasensor for IgE detection with a LOD of 500 pM. Machashi *et al.*³⁸ fabricated a label-free aptasensor based on aptamer-modified carbon nanotube field-effect transistors (CNT-FETs) for the detection of IgE with a LOD of 250 pM. In order to function in real-world applications, a sensor must achieve acceptable sensitivity, specificity, and selectivity. Although all the above methods have demonstrated specific IgE detection with practically useful working ranges, an ultrasensitive optical aptasensor will be a useful analytical tool for direct detection of IgE in human serum because of its complex composition.

Table 1 Comparison of sensitivity for different IgE assay methods

Analytical method	Label	Detection limit
CE-LIF	FITC	46 pM ³⁹
Fluorescence	Fluorescence dyes	10 ng mL ⁻¹ ⁴⁸
Fluorescence	Cy3	0.01 nM ⁴⁹
Fluorescence	Label-free	100 pM ⁵⁰
Fluorescence	Texas red/Dabcyl	57 pM ⁵¹
Fluorescence polarization	Texas red/Fluorescein	350 pM ⁴⁰
MALDI-MS	Label-free	100 fM–1 pM ⁴¹
Electrochemical	Pyrroquinoline quinone glucose dehydrogenase	1 nM ⁵²
Electrochemical	Label-free	36 pM ⁵³
Electrochemical	Alkaline phosphatase (AP)	0.02 nM ⁴²
Electrochemical	AP	23 ng mL ⁻¹ ⁵⁴
Electrochemical	Label-free	0.1 nM ⁵⁵
CNT-FET	Label-free	250 pM ³⁸
CNT-FET	Label-free	250 pM ⁵⁶
QCM	Label-free	0.5 nM ³⁷
QCM	Label-free	2.5 µg L ⁻¹ ⁵⁷
Surface plasmon resonance (SPR)	Au NPs	1 ng mL ⁻¹ ⁵⁸
SPR	Label-free	2.07 ng mL ⁻¹ ⁵⁹
SPR	Label-free	2 nM ⁶⁰
Clotting	Label-free	250 nM ⁶¹
CL	Au NPs	50 fM (9.5 pg mL ⁻¹)

Compared with the above-mentioned methods, the detection limit of the present method is significantly increased to be 50 fM with a 5000-fold improvement in amplification, thereby surpassing the limits of previous capillary electrophoresis (CE),³⁹ fluorescence anisotropy,⁴⁰ affinity MALDI-MS,⁴¹ and enzyme-electrochemical methods.⁴² Moreover, the present technique combines this unprecedented sensitivity and selectivity in a single approach, rendering it possible to detect physiologically relevant IgE levels directly in human serum samples.

Experimental

Materials

All chemicals were of analytical reagent grade and were used as received. Water was purified using a Millipore Milli-XQ system (Bedford, MA). DNA-BIND 96-well plates were obtained from Corning Incorporated. 40 nm streptavidin gold was bought from BB International. 10 nm streptavidin gold, monoclonal anti-human IgE and streptavidin-HRP were bought from Sigma-Aldrich. Human IgE protein was purchased from Abcam (Hong Kong) Ltd. HRP substrate kits were purchased from Millipore Corporation, USA. Bovine serum albumin (BSA) was bought from Sino-American Biotechnology Co. Particle-enhanced turbidimetric immunoassay (PETIA) reagent kits for IgE were provided by Shanghai Shenergy-Diasys Diagnostic Technology Co. Ltd., China. NH₂OH, HAuCl₄, and other chemical reagents were purchased from Sinopharm Chemical Reagent Co. Ltd. Oligonucleotides were acquired from Invitrogen Biotechnology Co., Ltd (Shanghai, China). Aptamer DNA: 5'-GGGGCAGCTT TATCCGTCCCTCCTAGTGGCGTGCCCC-biotin-3'.

Apparatus

Chemiluminescence (CL) measurements were carried out using a PC-controlled Fluoroskan Ascent FL (Thermo Electron Corporation). Absorbance was determined by a HITACHI U-2900 spectrophotometer. Sizes and morphologies of Au NPs were determined at 200 kV using a JEOL JEM-2010F high-resolution transmission electron microscope (HR-TEM).

Preparation of aptamer-functionalized Au NPs

80 pmol biotinylated aptamer sequences were added to 200 μL of PBS (8 mM Na_2HPO_4 , 2 mM NaH_2PO_4 , pH 7.4, 0.9% NaCl) solution that contained 48 μL of 40 nm streptavidin gold. The mixture was incubated at 37 $^\circ\text{C}$ for 1 h. The conjugates were washed several times with 200 μL PBS-TW wash buffer (8 mM Na_2HPO_4 , 2 mM NaH_2PO_4 , pH 7.4, 0.9% NaCl, 0.05% Tween 20) by centrifuging at 12000 rpm for 3 min. The soft sediment of gold probes was then resuspended in 80 μL PBS containing 1% BSA at 4 $^\circ\text{C}$ before use. The concentration of gold probes was estimated by UV-Vis spectroscopy to be about 0.67 nM, based on an extinction coefficient of $9.264 \times 10^9 \text{ M}^{-1} \text{ cm}^{-1}$ at $\lambda = 520 \text{ nm}$ for 40 nm Au NPs.

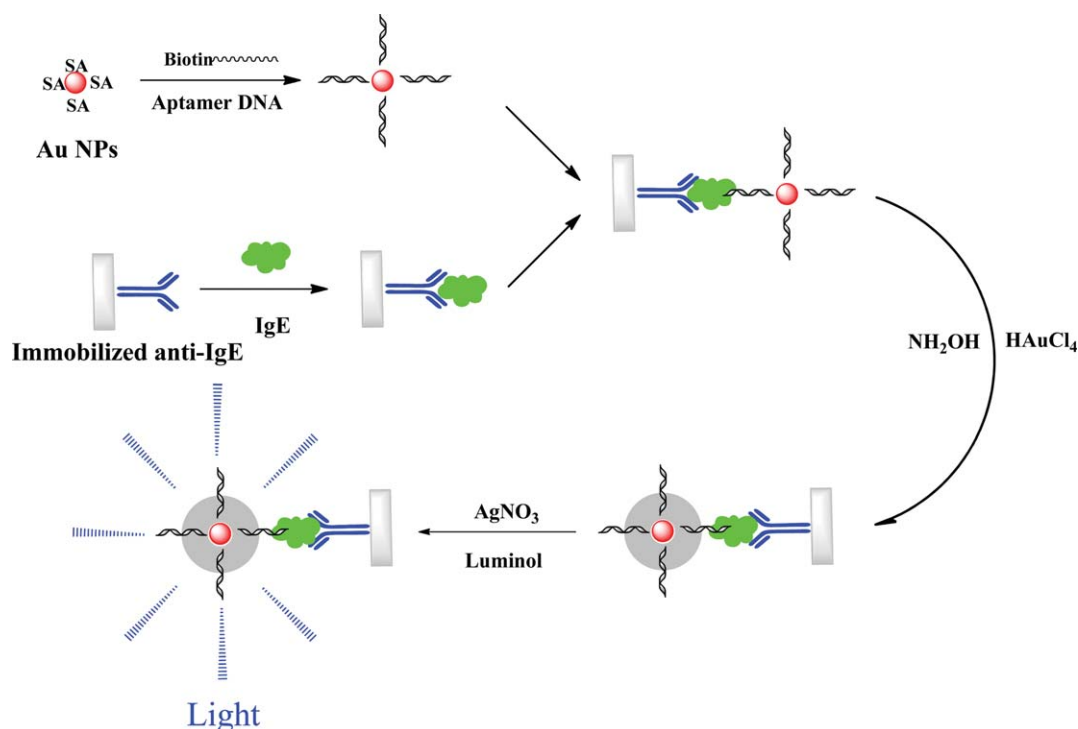
Assay procedures on polystyrene microwells

In a typical experiment, anti-human IgE was diluted to 8 μg per 100 μL of coupling buffer (0.5 M $\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$, pH 8.5) and divided into the wells of a DNA-BIND 96-well plate, 100 μL per well). The wells were washed three times with wash buffer after incubating with gentle mixing for 1 h at 37 $^\circ\text{C}$. Then, the wells were blocked with blocking buffer (PBS containing 3% BSA, 150 μL per well) at 37 $^\circ\text{C}$ for 1 h. After decantation of

blocking buffer, different concentrations of human IgE or nontarget protein was then added into each well (100 μL per well, in PBS). Following a 1 h incubation with gentle mixing at 37 $^\circ\text{C}$, the wells were washed three times with wash buffer. Then 10 μL gold probes were added and incubated in 100 μL PBS containing 1% BSA at 37 $^\circ\text{C}$ for 1 h. The wells were then washed three times with wash buffer before 50 μL of 1 mM luminol (0.1 M NaOH) was pipetted into the microwells. Finally, 50 μL of 0.2 mM AgNO_3 solution was injected and the CL signal was displayed in the Fluoroskan Ascent FL. For the amplification assay procedure, Au NPs that assembled on the surface of the 96-well plate were catalytically enlarged in the presence of 1 mM NH_2OH and 0.1 mM HAuCl_4 at 25 $^\circ\text{C}$ for 10 min. The wells were washed three times with wash buffer, and then detected as described above. For comparison, streptavidin-HRP was also used instead of gold probes, and CL signals on the surface of 96-well plate were detected directly with 100 μL of CL HRP substrate.

Results and discussion

Scheme 1 shows the principle behind the method used here, which involves the following three steps: (1) monoclonal anti-IgE antibodies are covalently immobilized on the surfaces of activated 96-well plates and reacted with IgE to initiate the antigen-antibody immunoreactions; (2) aptamer-functionalized Au NPs are added to sandwich to the surface of the 96-well plate to form a ternary complex; (3) Au NPs thus assembled on the surface of the 96-well plate are reacted with HAuCl_4 and NH_2OH , and the gold metal is catalytically deposited onto the surfaces of the Au NPs and the CL signal is detected directly with a simple and direct luminol- AgNO_3 CL reaction^{43–45} through a non-stripping Au NPs catalysis.



Scheme 1 Schematic representation of ultrasensitive detection of immunoglobulin E by non-stripping aptamer-functionalized Au NPs.

It is well known that the strong reductive activity of NH_2OH resulted in the rapid formation of small Au particles with a diameter around 3 nm in the growth solution. Subsequent attachment of the small Au particles onto the seed surface contributed to the production of larger Au particles.^{31,46} Herein the Au NPs were immobilized on the 96-well plate surface through the specific interaction between aptamer conjugated Au NPs and target IgE reacted on the anti-IgE modified 96-well plate surface. These immobilized Au NPs were enlarged by 0.1 mM HAuCl_4 and 1 mM NH_2OH . Consequently, the staining CL signals were considerably enhanced. To examine the benefits of enhancing sensitivity, 40 nm Au NPs were used to study the increase of the diameter and CL intensity of the Au NPs induced by the enlargement with HAuCl_4 and NH_2OH . The diameters of the Au NPs were increased to be around 290 nm and 580 nm after single and double enlargement, respectively. Note also that Au NPs were released from the 96-well plate surface in the 0.1 M glycine solution (pH 2.0) for TEM measurement. Compared with the CL intensity of 40 nm Au NPs without enlargement, these results mean that the CL intensities of the Au NPs were remarkably increased approximately 8-fold and 30-fold (Fig. 1).

Optimization of reaction parameters

Several parameters were investigated systematically to establish optimal conditions for the CL detection of the IgE, including the amounts of anti-IgE, biotinylated aptamer DNA, and 40 nm streptavidin gold.

As shown in Fig. 2, with the increase of the amount of anti-IgE, the CL intensity was observed to increase over the range of 0–8 μg of anti-IgE and then remained almost constant, possibly due to the binding sites on the wells being progressively saturated

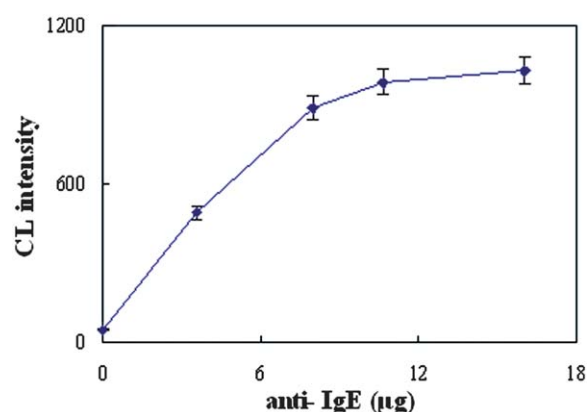


Fig. 2 CL intensity vs. the amounts of anti-IgE. Experimental conditions: IgE and biotinylated aptamer DNA were 20 fmol and 10 pmol, 40 nm streptavidin gold was 33.5 pM, NH_2OH , HAuCl_4 , luminol and AgNO_3 were 1, 0.1, 2.5 and 0.1 mM, respectively, Au enlargement time was set to be 10 min. The detection procedure was carried out as described in the Experimental section.

and thus no more sandwiched complex was formed on the wells. Thus, 8 μg of anti-IgE was selected for subsequent experiments.

The effects of the amounts of biotinylated aptamer DNA and streptavidin-gold were subsequently examined and optimized. First, the CL signal intensity increased with increasing concentration of biotinylated aptamer DNA over the range of 1 to 10 pM, and then slowly decreased in the range of 10–50 pM (Fig. 3), possibly due to the more tightly packed aptamer DNA on the streptavidin gold surface limiting access to the surface-bound aptamer DNA by the target protein. The CL intensity increased in the range of 11–67 pM streptavidin-gold and then

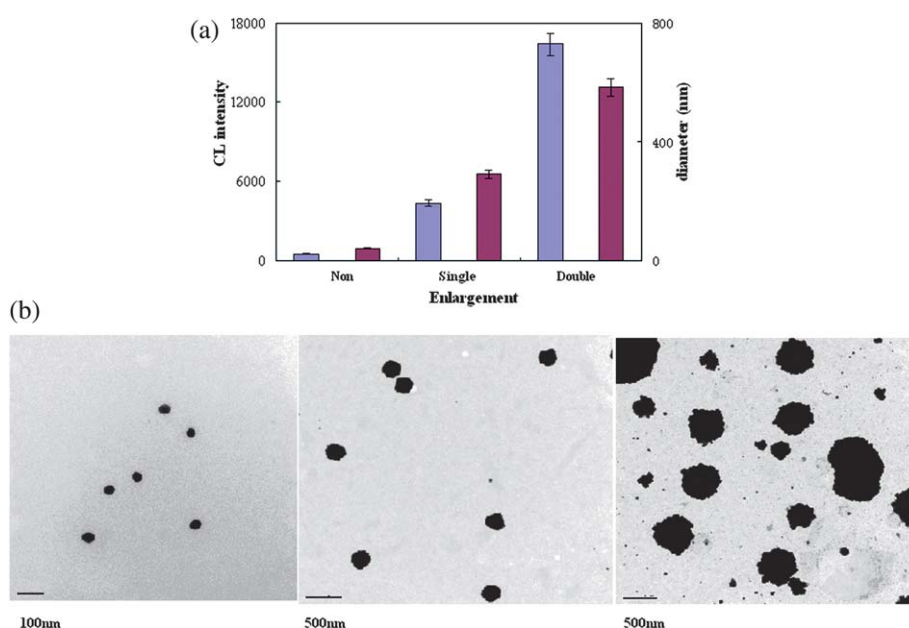


Fig. 1 Diameter (brown) and CL intensity (blue) of Au NPs with and without enlargement (top) and TEM images without enlargement, with single and double enlargement (bottom, from left to right), respectively. Experimental conditions: anti-IgE was 8 μg , IgE and biotinylated aptamer DNA were 20 fmol and 10 pmol, 40 nm streptavidin gold was 67 pM, NH_2OH , HAuCl_4 , luminol and AgNO_3 were 1, 0.1, 1 and 0.2 mM, respectively, Au enlargement time was set to be 10 min. The detection procedure was carried out as described in the Experimental section.

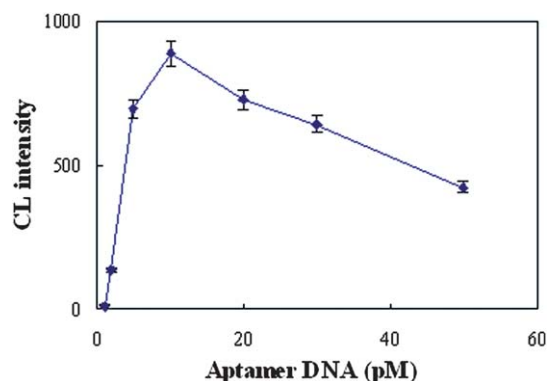


Fig. 3 CL intensity vs. the amounts of aptamer DNA. Experimental conditions: anti-IgE was 8 μ g, IgE was 20 fmol, 40 nm streptavidin gold was 33.5 pM, NH_2OH , HAuCl_4 , luminol and AgNO_3 were 1, 0.1, 2.5 and 0.1 mM, respectively, Au enlargement time was set to be 10 min. The detection procedure was carried out as described in the Experimental section.

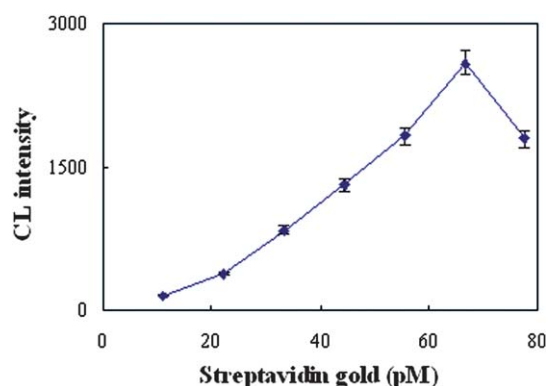


Fig. 4 CL intensity vs. the amounts of streptavidin gold. Experimental conditions: anti-IgE was 8 μ g, IgE and biotinylated aptamer DNA were 20 fmol and 10 pmol, NH_2OH , HAuCl_4 , luminol and AgNO_3 were 1, 0.1, 2.5 and 0.1 mM, respectively, Au enlargement time was set to be 10 min. The detection procedure was carried out as described in the Experimental section.

decreased (Fig. 4). Considering the decrease of the CL intensity at concentration larger than 67 pM streptavidin-gold, these results suggest that the number of the aptamer DNA per particle began to decrease from the excess of streptavidin gold, thus leading to a decrease in the CL intensity.

The effects CL intensity and ratio was used to monitor the Au growth as a function of growing time (Fig. 5). The particle growth initiated as soon as the addition of growth solution. The CL intensity was sharply increased over the range of 2–10 min, and then slowly increased and reached a maximum and finally decreased after 20 min. However, a maximum CL ratio (signal/blank) was found to be at 10 min. Thus, the enlargement reaction is selected to be complete at 10 min.

In addition, the concentrations of luminol and AgNO_3 also affected the CL signal. Therefore, these parameters were also examined and optimized. First, it was found that CL intensity increased with increasing luminol concentration, reached a maximum and then decreased slowly after a concentration of

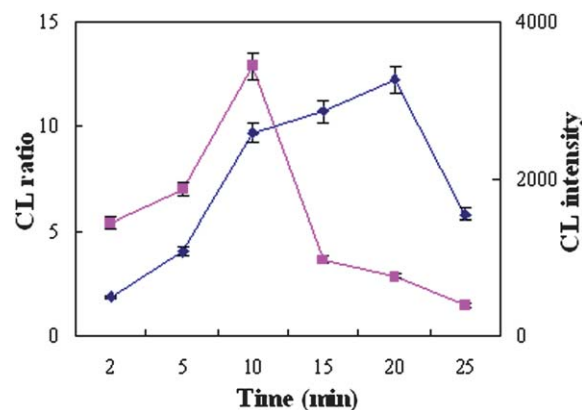


Fig. 5 CL ratio (pink) and intensity (blue) vs. Au enlargement time. Experimental conditions: anti-IgE was 8 μ g, IgE and biotinylated aptamer DNA were 20 fmol and 10 pmol, 40 nm streptavidin gold was 67 pM, NH_2OH , HAuCl_4 , luminol and AgNO_3 were 1, 0.1, 2.5 and 0.1 mM, respectively. The detection procedure was carried out as described in the Experimental section.

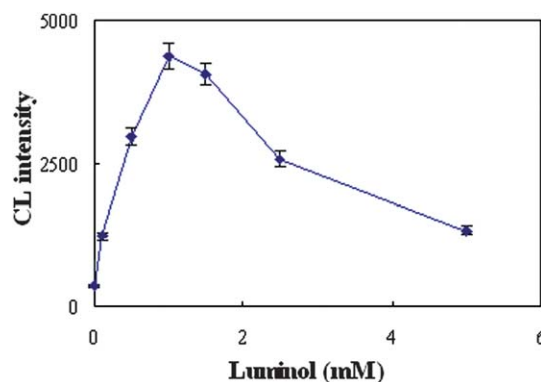


Fig. 6 CL intensity vs. the amounts of luminol. Experimental conditions: anti-IgE was 8 μ g, IgE and biotinylated aptamer DNA were 20 fmol and 10 pmol, 40 nm streptavidin gold was 67 pM, NH_2OH , HAuCl_4 and AgNO_3 were 1, 0.1 and 0.1 mM, respectively, Au enlargement time was set to be 10 min. The detection procedure was carried out as described in the Experimental section.

1 mM (Fig. 6). We attribute this decrease in the CL quenching possibly caused from an excess of luminol, where the excitation energy from the excited state 3-aminophthalate was lost by non-radiative pathways. For AgNO_3 , the CL intensity increased over the range of 0.01–0.2 mM, and then decreased (Fig. 7), possibly because AgNO_3 at higher concentrations would form precipitates that covered the surface of Au NPs and thus decreased its catalytic ability in the CL reaction between luminol and AgNO_3 .⁴³ Hence, 1 mM luminol and 0.2 mM AgNO_3 were selected for subsequent experiments.

Au NPs-based Au amplification for the quantification of target IgE

Under the above-optimized experimental conditions, the quantitative behavior of the method was assessed by monitoring the dependence of the CL intensity upon the concentration of IgE. A calibration graph in the concentration range of 0.2–1000 pM

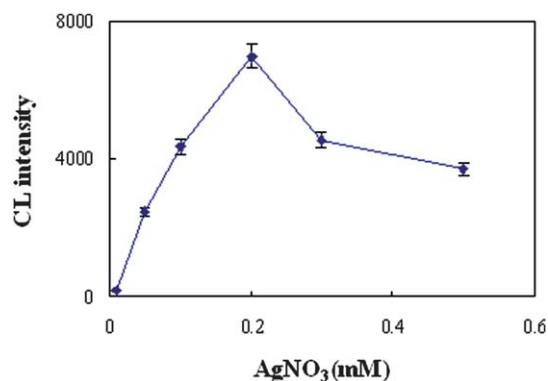


Fig. 7 CL intensity vs. the amounts of AgNO_3 . Experimental conditions: anti-IgE was 8 μg , IgE and biotinylated aptamer DNA were 20 fmol and 10 pmol, 40 nm streptavidin gold was 67 pM, NH_2OH , HAuCl_4 and luminol were 1, 0.1 and 1 mM, respectively, Au enlargement time was set to be 10 min. The detection procedure was carried out as described in the Experimental section.

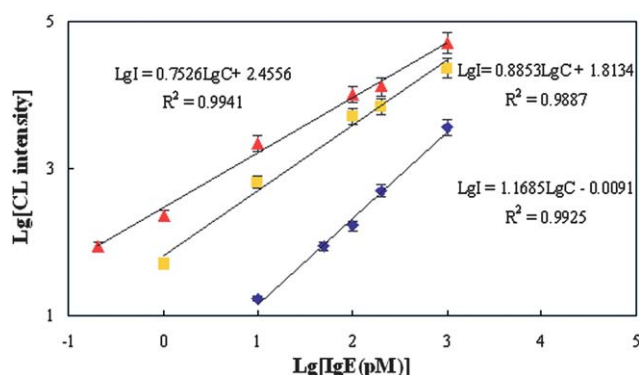


Fig. 8 Log-Log calibration data without (blue) and with single (yellow) and double (red) enlargement. Experimental conditions: anti-IgE was 8 μg , biotinylated aptamer DNA was 10 pmol, 40 nm streptavidin gold was 67 pM, NH_2OH , HAuCl_4 , luminol and AgNO_3 were 1, 0.1, 1 and 0.2 mM, respectively, Au enlargement time was set to be 10 min. The detection procedure was carried out as described in the Experimental section.

showed an approximately linear correlation ($R^2 = 0.9941$, Fig. 8) between the concentration of IgE and the CL intensity after double enlargement (represented by $\text{Lg}I = 0.7526\text{Lg}C + 2.4556$, where I is the CL intensity and C is the concentration of IgE). The detection limit was estimated as 50 fM from three times the standard deviation corresponding to the blank sample detection. Taking the sample volume into account, we detected about 5 amol of protein molecules. The sensitivity of our assay compares favorably with previous efforts using the same IgE aptamer (Table 1); for example, a CE-based technique offered a detection limit of 46 pM in serum samples³⁹ while an electrochemical approach yielded a detection limit of 20 pM in PBS solution.⁴² By using a QCM-based detection scheme, Liss *et al.* detected IgE with a LOD of 500 pM in spiked milk and meat extract samples,³⁷ and 350-pM detection limits were obtained by fluorescence anisotropy.⁴⁰ Note also that an affinity MALDI-MS aptameric assay could achieve detection of IgE at concentrations of 100 fM after 10 successive incubation/rinse cycles,⁴¹ however,

this method cannot be used for quantitative measurements. A series of six repetitive measurements of 10, 100 and 1000 pM IgE were used for estimating the precision and yielded relative standard deviations of 1.61%, 1.50% and 5.77%, respectively. To further improve the sensitivity of our assay, we also tried triple enlargement or more, however it was found that CL intensity and the detection limit was almost the same by either double enlargement or more.

Meanwhile, we also employed 10 nm streptavidin gold. For 10 nm streptavidin gold, in the concentration of 50–40000 pM, there was a linear correlation ($R^2 = 0.9925$, Fig. 9) between the concentration of target IgE and the CL intensity (represented by $\text{Lg}I = 0.5677\text{Lg}C + 0.7437$, where I is the CL intensity and C is the concentration of target IgE). The detection limit was estimated to be 10 pM (3σ , $n = 5$) for 10 nm streptavidin gold without enlargement. After single enlargement, the detection limit was further improved to be 1 pM (3σ , $n = 5$) for 10 nm streptavidin gold. However, the detection limit could reach 1 pM for 40 nm streptavidin gold even without enlargement. We assume that this was associated with the size of the streptavidin gold, and 40 nm streptavidin gold exhibited a much higher catalysis activity than 10 nm streptavidin gold.

Moreover, the CL intensity in our experiments was decided by two factors, *i.e.* particle size and particle number. In our case, since our target IgE concentration was comparatively very low, possibly the number of Au NPs combined on the 96 wells plates were almost the same, no matter whether 10 nm or 40 nm Au NPs were used, leading to a higher CL intensity being seen for 40 nm Au NPs than 10 nm ones. As reported before, larger particle probes can improve assay sensitivities, but sedimentation becomes more dominant with the increase in the particle size,⁴⁷ thus 40 nm streptavidin gold was used in this research.

For comparison, we also carried out conventional CL enzyme linked oligonucleotide assay (ELONA) on the surface of the 96-well plate as shown in Fig. 10. A linear correlation was also observed in the concentration of 500–40000 pM between the concentration of target IgE and the CL intensity (represented by

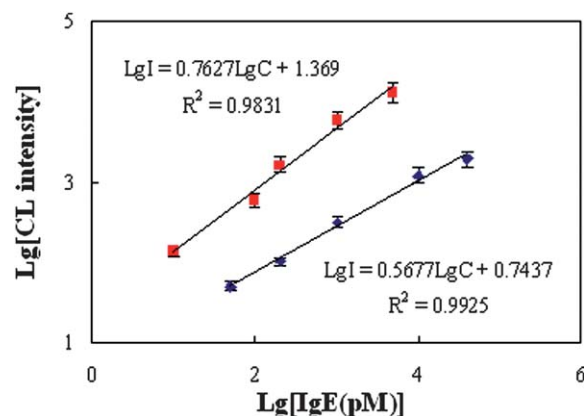


Fig. 9 Log-Log calibration data without (blue) and with single (red) enlargement. Experimental conditions: anti-IgE was 8 μg , biotinylated aptamer DNA was 10 pmol, 10 nm streptavidin gold was 67 pM, NH_2OH , HAuCl_4 , luminol and AgNO_3 were 1, 0.1, 1 and 0.2 mM, respectively, Au enlargement time was set to be 10 min. The detection procedure was carried out as described in the Experimental section.

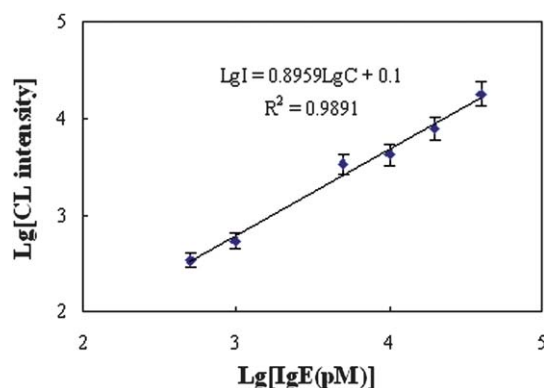


Fig. 10 Log-Log calibration data. Experimental conditions: anti-IgE, biotinylated aptamer DNA and streptavidin-HRP were 8 μ g, 10 pmol and 20 pg, respectively. The detection procedure was carried out as described in the Experimental section.

$LgI = 0.8959LgC + 0.1$, $R^2 = 0.9891$, where I is the CL intensity and C is the concentration of target IgE). The detection limit of conventional CL ELONA was 400 pM, which was much higher than our method by one to 4 orders of magnitude.

Assay specificity

To address the nonspecific adsorption of the non-IgE aptamer-functionized Au NPs on the 96-well plate surface control experiments were performed. Non-IgE aptamer-functionized Au NPs were added to sandwich to the surface of the 96-well plate to form a ternary complex after blocking with 3% bovine serum albumin (BSA). The optical density of the Au NP was the same as that of the conjugate of aptamer-functionized Au NPs. The 96-well plate surface was treated with the mixture of 0.1 HAuCl₄ and 1 mM NH₂OH for 10 min each time at 25 $^{\circ}$ C, rinsed with wash buffer, and then treated with fresh solution for another 10 min at 25 $^{\circ}$ C. Detectable staining caused by the nonspecific adsorption of non-IgE aptamer-functionized Au

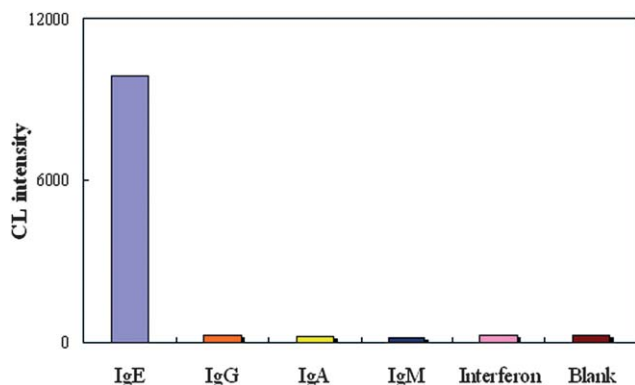


Fig. 11 Assay specificity. The samples tested were: 500 pM IgE, IgG, IgA, IgM, interferon, and a negative control containing no protein. Experimental conditions: anti-IgE was 8 μ g, biotinylated aptamer DNA was 10 pmol, 40 nm streptavidin gold was 67 pM, NH₂OH, HAuCl₄, luminol and AgNO₃ were 1, 0.1, 1 and 0.2 mM, respectively, Au enlargement time was set to be 10 min. The detection procedure was carried out as described in the Experimental section.

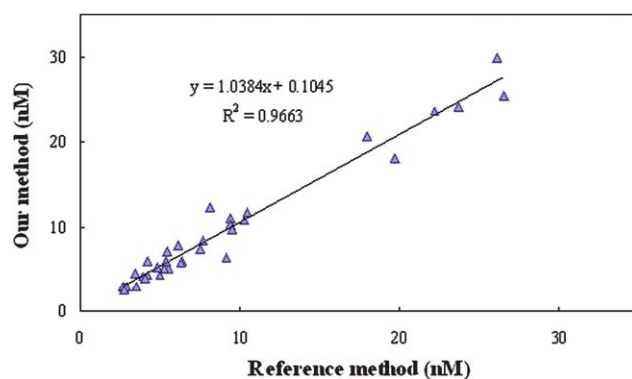


Fig. 12 Relationship between the proposed method and the immunoturbidimetric assay for IgE. Experimental conditions: anti-IgE was 8 μ g, biotinylated aptamer DNA was 10 pmol, 40 nm streptavidin gold was 67 pM, NH₂OH, HAuCl₄, luminol and AgNO₃ were 1, 0.1, 1 and 0.2 mM, respectively, Au enlargement time was set to be 10 min. The detection procedure was carried out as described in the Experimental section.

NPs could not be observed. In contrast, the CL response of IgG, IgA, IgM and interferon was examined, all at a concentration of 500 pM. The ratio of the CL intensities of the five antigens was 100 : 2.9 : 2.5 : 2 : 2.9, respectively (Fig. 11). Hence, good selectivity can be obtained using this new CL aptasensor. These results demonstrate that neither nonspecific binding of Au NPs nor nonspecific new gold nucleation formed on the 96-well surface blocked with BSA.

Comparison with immunoturbidimetric assay

We then challenged our method with 35 human serum samples from normal individuals and patients. Samples were taken and divided into two aliquots, one for our method and one for the immunoturbidimetric test by Zhongshan Hospital. As can be seen from Fig. 12, the distributions of IgE levels in 35 serum samples were approximated by a normal distribution. The correlation was calculated with a linear least-squares method, and the 95% confidence intervals of the slope and intercept were 0.96852 to 1.10827 and -0.68164 to 0.89064 for IgE, respectively. The confidence intervals for the slope and intercept values include the unit and zero values, respectively; thus, the results indeed showed no significant difference between the conventional assay and the proposed one, as confirmed by the Student *t*-test ($P > 0.05$). Moreover, the multiplexed detection of several proteins in an array format is currently being explored.

Conclusions

In summary, enlarging Au NPs immobilized on the 96-well surface was performed with a mixture of 0.1 mM HAuCl₄ and 1 mM NH₂OH instead of silver staining to detect human IgE, as a result, the detection sensitivity was significantly increased to approximately 50 fM. This level is lower by one to 3 orders of magnitude than the limits of fluorescent, QCM, and enzyme-electrochemical methods. From the analytical chemistry point of view, the protocol can be readily extended to a large variety of clinical markers such as platelet-derived growth factor-BB (PDGF). The data show that PDGF could also be sensitively

detected with a detection limit of 10 fM. Note also that this design of the aptasensor requires the immobilization of the corresponding antibody on a surface for capture of aptamer functionalized AuNPs. Therefore, this method applies only to those targets with a known corresponding antibody. In addition, the aptasensor will not work well if the binding of the antibody to the target protein interferes with the binding of the aptamer to the protein. Overall, the method described is simple, rapid, economic, sensitive, specific, and appears well suited for the direct detection of proteins in complex, unprocessed clinical samples. It also provides a realistic possibility of aptamer-based applications for convenient point-of-care diagnostics and the field monitoring of proteins, and these current results suggest a new way in immunoassay.

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