

Cite this: *Analyst*, 2011, **136**, 4295

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PAPER

Highly sensitive rapid chemiluminescent immunoassay using the DNAzyme label for signal amplification

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Received 21st June 2011, Accepted 26th July 2011

DOI: 10.1039/c1an15512a

A novel trace tag for chemiluminescent (CL) immunoassay was designed by using DNAzyme to functionalize antibody-labeled Au nanoparticles (AuNPs). The trace tag showed an excellent ability to catalyze the oxidation of luminol by hydrogen peroxide, leading to strong CL emission. By coupling the trace tag with a passive mixing accelerated immunoreaction system, a highly sensitive rapid flow-through CL immunoassay method was proposed. Using carcinoembryonic antigen (CEA) as a model analyte, the capture antibody for CEA was immobilized on paramagnetic microspheres, and DNAzyme-anti-CEA antibody functionalized AuNPs were prepared as trace tag. A three-dimensional helical glass tube kept at 37 °C in a water bath was used for passively mixing immunoreagents in a two-step sandwich immunoassay, with which each immunoreaction step could be finished within 150 s. With the help of a magnet, the immunocomplex could conveniently be separated from reactants. Compared with the horseradish peroxidase-based tag, the newly designed trace tag showed obvious signal amplification due to its strong catalytic ability and high loading ratio of DNAzyme on each AuNP. The proposed method showed a linear calibration range from 0.005 to 0.5 ng mL⁻¹ for CEA detection with a detection limit of 4.1 pg mL⁻¹ at a signal-to-noise ratio of 3 and acceptable detection reproducibility. The assay results of clinical serum samples were in acceptable agreement with the reference values. The designed immunoassay system with ultrahigh sensitivity provided a programmable and low-cost approach for high-throughput clinical application.

Introduction

Highly sensitive and selective detection of biomarkers has been showing its significance in early screening of disease, evaluating the extent of disease, and monitoring the response of disease to therapy.¹ Many natural enzymes such as horseradish peroxidase (HRP) and alkaline phosphatase have been extensively used as biological catalysts to produce detection signals. By functionalizing nanoparticles with these enzymes for preparation of trace tags, various highly sensitive detection methods have been proposed.^{2–4} However, natural enzymes can usually lose their catalytic activity due to minor alteration in their conformation during storage. The high cost for purifying these natural enzymes also limits their application. Furthermore, the catalytic activity of some natural enzymes is often limited due to the embedding of the activity center in macromolecular peptides. Thus great effort has focused on the development of artificial enzyme mimics, which offer new opportunities to achieve catalysis with high operational stability and reusability. Several peroxidase mimics

such as hemin,⁵ Fe₃O₄ nanoparticles,⁶ Au nanoparticles (AuNPs),⁷ and DNAzyme^{8–10} have been applied in bioanalysis. Among these mimics, hemin/G-quadruplex DNAzyme formed by embedding a hemin in a guanine-rich DNA strand has attracted considerable attention due to its high catalytic ability as HRP.^{8–10} Using this DNAzyme as catalytic label, several bio-analytical methods have been developed for genotyping of single nucleotide polymorphism⁸ and detection of low-molecular-weight substrates, DNA or even protein.⁹ In order to amplify the catalytic ability and achieve high detection sensitivity, AuNPs have been used as DNAzyme carrier for chemiluminescent (CL) detection of DNA and telomerase activity.¹⁰ This work designed a novel trace tag for CL immunoassay by using DNAzyme to functionalize antibody-labeled AuNPs.

Owing to the high surface-to-volume ratio and good biocompatibility, AuNPs have been extensively used as HRP carrier for preparation of trace tags. These HRP functionalized AuNPs show excellent signal amplification in both CL and electrochemical immunoassays.^{2,3} By binding hemin with a guanine-rich DNA strand, which was disassociated from DNA functionalized AuNPs linked to an immunocomplex, to form peroxidase-mimicking DNAzyme, Zhou *et al.*¹¹ developed an enzyme-linked immunosorbent assay (ELISA) for tumor markers. This ultraviolet adsorption method showed a detection limit of 0.1 ng mL⁻¹ for α -fetoprotein, which was obviously insufficient for detection of low-abundant

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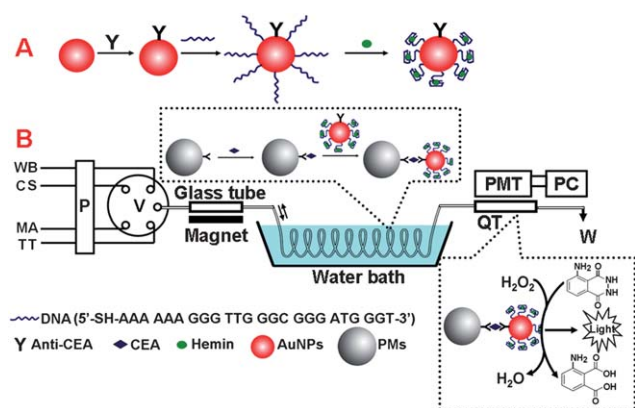
proteins. Here, a simple CL immunoassay method was proposed by directly forming DNAzyme on signal antibody functionalized AuNPs for immunoreaction and using the attached DNAzyme to catalyze the oxidation of luminol by hydrogen peroxide for producing amplified CL emission (Scheme 1).

CL immunoassay, which combines good specificity of immunoreaction with high sensitivity of CL detection, has been well known as a powerful and important technique.^{12,13} However, due to the low diffusive rate of macromolecular immunoreagents, the detection procedure generally needs long incubation time,^{13,14} which may lead to absorption of immunoreagents on the channel inner wall and thus cross talk among the tests of different samples in a one-way fluidic system. To overcome this drawback and achieve high-throughput immunoassay, different ways such as electrokinetic flow,¹⁵ micro-bubble,¹⁶ and magnetic stirring¹⁷ have been employed to accelerate the immunoreaction. Previously, we proposed a dual acceleration strategy using infrared heating and passive mixing to develop a rapid flow-through CL immunoassay (FTCLIA) method.¹⁸ A three-dimensional helical glass tube was used for rapid mixing and the immunoassay process could be completed within 3 min. The present work further simplified the incubation process by replacing the infrared heating with a water bath at 37 °C (Scheme 1B) and amplified the CL signal with the newly designed AuNP-based multi-DNAzyme trace tag. Using carcinoembryonic antigen (CEA) as a model analyte, each immunoreaction step could be finished within 150 s and the whole assay could be finished within 8 min. Compared with the HRP-based tag, the DNAzyme-antibody functionalized AuNPs produced stronger CL emission, leading to a highly sensitive FTCLIA with a detection limit down to 4.1 pg mL⁻¹. This proposed method provided a promising platform for practical clinical detection of serum tumor markers with high throughput.

Experimental

Materials and reagents

Mouse monoclonal capture (clone no. D0102-1) and signal anti-CEA antibodies (clone no. D0102-2) were purchased from



Scheme 1 (A) Schematic preparation of DNAzyme-antibody functionalized AuNPs. (B) Schematic diagram of the rapid sandwich FTCLIA system: (WB) washing buffer, (CS) CL substrate, (MA) mixture of antibody functionalized PMs and antigen, (TT) DNAzyme and antibody functionalized AuNPs trace tags, (P) pump, (V) injection valve, (QT) quartz tube, and (W) waste.

Shuangliu Zhenglong Biochem. Lab (China). DNA (5'-HS-AAAAAAGGGTTGGGCGGGATGGGT-3') and DNA which cannot form DNAzyme (5'-HS-TTTTTTTTTTCCCAACCCGCC-3') were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (China). Hemin was purchased from Sigma (St Louis, MO). Hemin stock solution (5 μM) was prepared in dimethyl sulfoxide and stored in the dark at 4 °C. CL ELISA kits of CEA, including standard solutions of CEA, and HRP substrate solutions (luminol-*p*-iodophenol and H₂O₂) were obtained from Autobio Diagnostics Co., Ltd. (China). Electrochemiluminescent immunoassay (ECLIA) reagent kits used for reference detection of CEA were supplied by Roche Diagnostics GmbH (Germany). HRP was obtained from Beijing Biosynthesis Biotechnology Co., Ltd. (China). Bovine serum albumin (BSA) was purchased from Nanjing Sunshine Bio Co., Ltd. (China). The aqueous suspension of paramagnetic silicon dioxide microspheres (PMs) coated with epoxy groups (diameter 0.5–1 μm and concentration 5 g L⁻¹) was obtained from Tianjin BaseLine Chromtech Research Centre (China). Ultrapure water obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore) was used in all runs. The clinical serum samples were obtained from Jiangsu Institute of Cancer Prevention and Cure. All other reagents were of the best grade available and used as received.

The coupling buffer for antibody immobilization was 0.01 M phosphate buffer solution (PBS, pH 7.4). The stock buffer was PBS containing 0.1% BSA. The blocking buffer was PBS containing 1% BSA. To minimize nonspecific adsorption, 0.05% Tween-20 was spiked into PBS (PBST) as the washing buffer. The regeneration buffer was 0.1 M glycine-HCl at pH 2.2.

Instrumentation

The Teflon tubes (0.8 mm i.d.) and silicon rubber tubes (1.0 mm i.d.) were used to connect all components in this system. All solutions were delivered with a multichannel bidirectional peristaltic pump of an IFFM-D Luminescent Analyzer (Remax, China). The introduction of different solutions into the one-way channel was performed using a multiposition valve of four inlets and one outlet. A heat concentrating magnetic mixer (DF101S, Jintan Medical Instrument Plant, China) was used for water bath incubation at 37 °C. The three-dimensional helical glass tube for incubation had an inner diameter of about 1 mm and 10 turns, and was coiled at a radius of about 12 mm, while the distance between two turns (the pitch) was about 8 mm. A permanent magnet provided the magnetic field to capture the PMs in a glass tube of 30 μL (length 4.0 cm, i.d. 1 mm), which was positioned in front of the helical glass tube followed by a quartz tube. The quartz tube was positioned in front of a photomultiplier (PMT) biased at -800 V to record the CL emission. The Instrument control and data record were performed using the IFFM software package run under Windows 2000.

The UV-vis spectroscopic experiment was performed with a UV-3600 UV-vis spectrophotometer (Shimadzu, Japan) to demonstrate the binding of monoclonal anti-CEA and thiolated DNA to AuNPs. An ESCALAB 250 spectrometer working in ultrahigh vacuum with an Al K-R X-ray source was employed for X-ray photoelectron spectroscopic (XPS) analysis of PMs before and after the immobilization of capture antibody. CL

spectral responses were recorded on a RF-5301 PC spectrofluorometer (Shimadzu, Japan). The reference ECLIA was performed with a Roche Elecsys 2010 immunoassay analyzer (Roche Diagnostics GmbH).

Preparation of DNAzyme-anti-CEA antibody functionalized AuNPs

The preparation of the DNAzyme-anti-CEA antibody functionalized AuNPs trace tag is illustrated in Scheme 1A. AuNPs (13 nm) were prepared according to the previously described procedure.¹⁹ The pH of colloidal gold solution was adjusted to 9.0 by adding 0.4 mM of NaOH, then 7 μL of 1 mg mL^{-1} of monoclonal anti-CEA was added to 1 mL of the solution. The mixture was incubated for 30 min at 10 $^{\circ}\text{C}$ under gentle stirring. Subsequently, 15 nmol of the guanine-rich DNA strand was added to the solution. After incubation for 16 h and then salt-stabilization with 0.1 M NaCl, 1 mL of 5% BSA solution was added with stirring for 30 min to passivate the left active sites on AuNPs. The resulting solution was centrifuged at 13 000 rpm at 4 $^{\circ}\text{C}$ for 20 min. The obtained functional AuNPs were suspended in 1 mL stock buffer at 4 $^{\circ}\text{C}$ for obtaining a concentration of 2.21×10^{12} nanoparticles mL^{-1} . Finally, hemin was added to the solution at a concentration of 500 nM and incubated at room temperature (RT) for 1 h. The solution of formed DNAzyme-anti-CEA antibody functionalized AuNPs was stored in a refrigerator at 4 $^{\circ}\text{C}$ for further use.

Preparation of anti-CEA immobilized PMs

The immobilization process of anti-CEA antibody on PMs was performed according to the previously described steps.²⁰ 1000 μL of PM suspension was washed with washing buffer three times using a permanent magnet to isolate them from the supernatant waste. The PMs were then resuspended in 1000 μL of coupling buffer and mixed with 20 μL of anti-CEA antibody solution (1 mg mL^{-1}) for covalent linking under gentle stirring at RT for 2 h and then in a refrigerator overnight. The resulting antibody immobilized PMs were thoroughly washed with washing buffer and then blocked with blocking buffer for 6 h. The antibody immobilized PMs were dispersed in PBS containing 0.01% NaN_3 to a volume of 1000 μL with a concentration of 5 g L^{-1} for storage at 4 $^{\circ}\text{C}$ and for all experiments.

Immunoassay protocol

The whole assay was controlled automatically by a personal computer equipped with the IFFM software package instead of manual manipulation (Scheme 1B). Anti-CEA antibody immobilized PMs were isolated from 30 μL of suspension, mixed with 30 μL of CEA, and then introduced into the helical glass tube. The mixture flowed back and forth at an optimal flow rate in the helical glass tube, which was immersed in a water bath at 37 $^{\circ}\text{C}$ for a selected incubation time of 2.5 min. After the first step incubation, the mixture flowed back into the glass tube to perform online separation of the immunocomplex immobilized PMs from free immunoreagents by a permanent magnet. Washing buffer was delivered at a flow rate of 0.8 mL min^{-1} into the system to wash away the free immunoreagents for 1 min. Next, the magnet was withdrawn, and 30 μL of

DNAzyme-antibody functionalized AuNPs was introduced into the glass tube to mix with CEA-anti-CEA antibody immobilized PMs. After the mixture was incubated in the helical glass tube for another 2.5 min using the same method, the formed sandwich immunocomplex was separated from free hemin and free DNAzyme-antibody functionalized AuNPs and washed in the glass tube with the help of a permanent magnet. Then the magnet was withdrawn, and 80 μL of CL substrate solution flowed fast into the tube to wash away the PMs and fully mix. Afterwards, the mixture flowed through the helical glass tube and was delivered into the quartz tube for recording the CL signal. This process takes 30 s to achieve complete reaction of the CL reagents. The whole procedure from sample injection to signal detection could be finished within 8 min. Finally, the one way detection channel was washed with 1 M HCl solution and washing buffer for 30 and 60 s, respectively, to avoid potential cross talk. After the detection, the sandwich immunocomplex immobilized PMs were expelled and collected, which could be regenerated by washing in regeneration buffer for 10 min and blocking buffer three times, respectively.

Results and discussion

Characterization of DNAzyme-anti-CEA antibody functionalized AuNPs and antibody immobilized PMs

UV-vis spectra were used to demonstrate the binding of the monoclonal anti-CEA and guanine-rich DNA strand to AuNPs (Fig. 1A). The size of AuNPs could be estimated to be 13 nm from the absorption peak at 519 nm (curve a). Compared with AuNPs, the UV-vis spectrum of antibody functionalized AuNPs showed a red shift of the absorption peak to 522 nm (curve b), indicating that a slight aggregation of the nanoparticles occurred after the binding of anti-CEA antibody onto AuNPs. After the guanine-rich DNA strand was attached on the surface of AuNPs, a strong absorption peak occurred at about 260 nm (curve c), which was obviously stronger than the absorption peak of anti-CEA antibody functionalized AuNPs at 280 nm (curve b) due to the adsorption of the guanine-rich DNA strand, indicating the successful binding of DNA to the surface of AuNPs.

PMs have been extensively used in immunoassays due to their advantages in separation and collection of the immunocomplex from free immunoreagents, which provides easy handling for automation analysis.²¹ This work used epoxy group modified

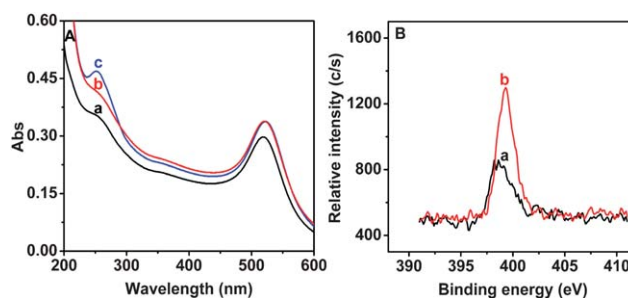


Fig. 1 (A) UV-vis absorption spectra of (a) AuNPs, (b) antibody functionalized AuNPs, and (c) DNAzyme-antibody functionalized AuNPs, and (B) XPS analysis of (a) PMs and (b) antibody immobilized PMs.

PMs with a diameter of 0.5–1 μm to immobilize the capture antibody. The XPS spectrum of the PMs showed an N (1s) peak at 399 eV (Fig. 1B, curve a), indicating the existence of nitrogen on the PM surface. After the capture antibody was covalently linked to the PMs, the height of the N (1s) peak at 399 eV increased by 2.2 times (curve b), which was obviously due to the covalent binding of the antibody on the surface of PMs.

Kinetics and mechanism of CL reaction

The kinetic behavior of the CL reaction catalyzed by DNAzyme labeled to the sandwich immunocomplex of CEA on PMs was studied with a static method. The CL reaction on PMs occurred immediately upon injection of the CL substrates. The intensity of CL emission increased quickly and reached its maximum value within 30 s. The CL kinetic of the DNAzyme system was obviously faster than the HRP system that reached the maximum value at 4 min.¹² This was in favor of the fast and high-throughput immunoassay. In order to acquire high detection sensitivity, 80 μL of CL substrate solutions flowed fast into the glass tube to fully mix up with the PMs, then the mixture flowed through the helical glass tube and was delivered into the quartz tube for recording the CL signal. This process from mixing to recording costs exactly 30 s, which matched the time reaching the maximum CL emission and thus helped the reagents to react completely for obtaining the sensitive CL signal.

A further inspection of CL spectra was performed on a spectrofluorometer (Fig. 2A). In the absence of hemin, the guanine-rich DNA could not fold to form a G-quadruplex structure, thus the CL intensity gave a very low response in the wavelength range from 370 to 500 nm (curve a). After addition of hemin into the guanine-rich DNA-antibody functionalized AuNPs, the formed G-quadruplex structure as DNAzyme led to a maximum emission peak at about 430 nm (curve b), which was at the same wavelength as that of the HRP-based tag (curve c), indicating the DNAzyme catalyzed CL process had the same CL reaction mechanism as the HRP catalyzed CL emission, in which the DNAzyme catalyzed the oxidation of luminol by hydrogen peroxide. It was interesting that at the same amounts of the

functionalized AuNPs and CL substrates the CL intensity of the DNAzyme catalyzed system (curve b) was much stronger than those of HRP-antibody functionalized AuNPs (curve c) and free hemin (curve d) systems, indicating the stronger catalytic ability of the DNAzyme-antibody functionalized AuNPs. The strong ability resulted from both the high catalytic activity of DNAzyme due to less embedding of the activity center in the G-quadruplex structure than in macromolecular peptides of HRP²² and the higher loading amount of DNAzyme on AuNPs due to its smaller size than HRP. The signal amplification phenomenon was further confined in the proposed FTCLIA with the CEA concentration of 0.05 ng mL^{-1} (Fig. 2B). These results suggested that the high catalytic ability of the DNAzyme-antibody functionalized AuNPs trace tag could lead to excellent signal amplification for highly sensitive immunoassay.

Incubation time and flow rate

To obtain high analytical performance, some important detection parameters, such as incubation time and flow rate, were optimized (Fig. 3). With the increasing incubation time, the CL signal increased and trended to the maximum value at 5 min (Fig. 3A), indicating the maximum formation of the sandwich immunocomplex. At an incubation time of 2.5 min, the CL signal was 80% of the maximum value, which was sufficient for obtaining high sensitivity. Considering the optimal analytical performance, 2.5 min was chosen as the optimal incubation time.

At the optimal incubation time the flow rate was further examined. With the increasing flow rate, the CL response increased and then quickly decreased, the maximum CL emission occurred at a flow rate of 10 $\mu\text{L s}^{-1}$ (Fig. 3B). At the flow rates higher than 10 $\mu\text{L s}^{-1}$, the constantly increasing variance and skewness led to less transversal mixing,²³ thus the CL response decreased. This work selected the flow rate of 10 $\mu\text{L s}^{-1}$ for immunoassay, with which the mixture of immunoreagents could flow back and forth in the helical tube ten times to match the optimal incubation time.

Regeneration of antibody immobilized PMs

Because of the covalent binding of the antibody to the PMs, the antibody immobilized PMs could be regenerated. By a simple and short-period dissociation of the sandwich immunocomplex, antibody immobilized PMs could be reused with little breaking of covalent bonds and little activity loss of immobilized antibody. The most efficient dissociating reagent was found to be

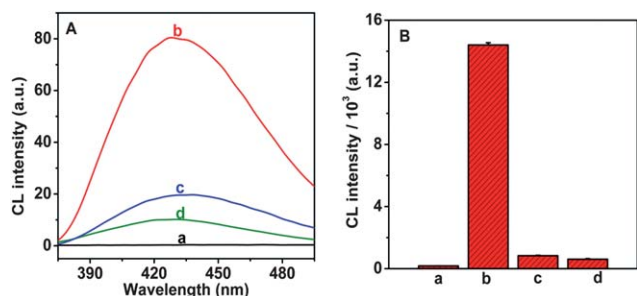


Fig. 2 (A) CL spectra recorded with an emission slit of 10 nm from 370 to 500 nm with an interval of 0.2 nm upon addition of 400 μL CL substrate into 50 μL (a) DNA-antibody functionalized AuNPs in the absence of hemin, (b) DNAzyme-antibody functionalized AuNPs, (c) HRP-antibody functionalized AuNPs and (d) 500 nM free hemin. (B) Comparison of the CL intensity at 0.05 ng mL^{-1} CEA using 30 μL of (a) DNA functionalized AuNPs without hemin, (b) DNAzyme functionalized AuNPs, (c) HRP-functionalized AuNPs to label the secondary antibody, and (d) control DNA functionalized AuNPs in the presence of hemin.

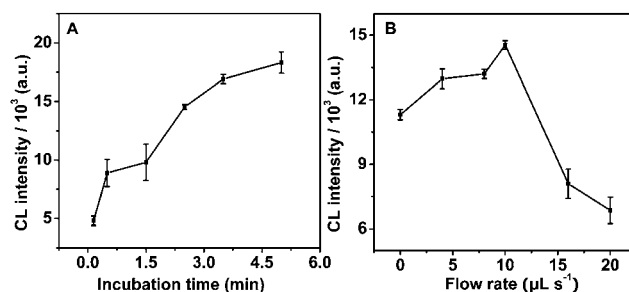


Fig. 3 Effects of (A) single incubation time and (B) flow rate on the CL intensity at 0.05 ng mL^{-1} CEA.

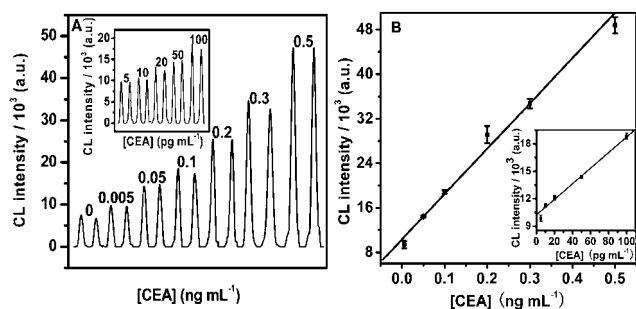


Fig. 4 (A) Dose–response curve and (B) calibration curve of the proposed method for CEA detection. Inset: dose–response curve and calibration curve in the low concentration range.

0.1 M glycine–HCl (pH 2.2), which allowed a fast and complete dissociation of the immunocomplex with a regeneration efficiency of 95%. As a mild regeneration reagent, the buffer showed good activity retention for the immobilized capture antibody. After the suspension of regenerated PMs in PBS containing 0.01% NaN_3 was stored for more than 20 days at 4 °C, no obvious change was observed.

Analytical performance

Under the optimized conditions, the dose–response curve and the calibration curve for immunoassay of CEA are shown in Fig. 4. The linear range for CEA detection was from 0.005 to 0.5 ng mL^{-1} with a relative coefficient of 0.996 and a detection limit of 4.1 pg mL^{-1} at 3 times the standard deviation of the blank response. The sensitivity was higher than those of gold nanolabel enhanced CL immunoassay methods.^{3,4} Since the cutoff value of CEA in diagnosis is 5 ng mL^{-1} , the practical clinical serum samples should be diluted with PBS at least 100 times prior to assay. More importantly, the proposed method with the DNAzyme-antibody functionalized AuNPs trace tag could be developed for immunoassay of low-abundant proteins.

The reproducibility of the proposed immunoassay system was assessed by the intra- and inter-assay coefficients of variation (CV) with a standard sample containing 0.05 ng mL^{-1} CEA for five time measurements. The obtained intra- and inter-assay CVs were 3.5% and 8.0%, respectively, showing acceptable detection and fabrication reproducibility.

When the DNAzyme-antibody functionalized AuNPs were not in use, they could be stored in stock buffer at 4 °C for 2 weeks without obvious signal change. The acceptable retention of

Table 1 Assay results of CEA in serum samples using the proposed and reference methods (in ng mL^{-1})

Sample	Proposed method ^a	Reference method	Relative error (%)
1	5.5	5.3	3.4
2	8.1	8.8	–8.2
3	62.0	56.6	9.6
4	226.7	218.7	3.7
5	259.2	251.4	3.1

^a The serum samples were diluted by 1 : 1000.

immunoreactivity is very important for the development of the proposed method in low-cost application.

Detection of CEA in serum samples

Under the optimum conditions, the analytical reliability and application potential of the proposed immunoassay system were evaluated by assaying clinical serum samples using the proposed method as well as the reference ECLIA method. The results are shown in Table 1, which indicate an acceptable agreement between the two methods, and the relative errors were less than 9.6%.

Conclusions

A novel DNAzyme amplified strategy coupled with a passive mixing accelerated FTCLIA system is proposed for ultrasensitive and rapid immunoassay. Compared with the HRP-based tag, the proposed DNAzyme-antibody functionalized AuNPs trace tag exhibits stronger catalytic ability and faster CL reaction kinetics, which result from both the high catalytic activity of DNAzyme due to less embedding of the activity center in the G-quadruplex structure than in macromolecular peptides of HRP and the higher loading amount of DNAzyme on AuNPs due to its smaller size than HRP. The DNAzyme-based signal amplification produces a much stronger CL response than HRP-based immunoassay at low analyte concentrations, thus leading to an ultrahigh sensitivity of CL immunoassay. During the incubation process the mixture can flow back and forth in the helical tube to maintain the passive mixing for accelerating the immunoreaction, so the whole assay including the incubation, washing and detection steps can be accomplished within 8 min. The immunosensing system shows a promising practicality in automated rapid screening and clinical diagnosis.

Acknowledgements

This work was supported by the National Science Fund for Creative Research Groups (20821063) and the projects (20875044, 21075055) from National Natural Science Foundation of China, and Natural Science Foundation of Jiangsu (BK2008014).

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