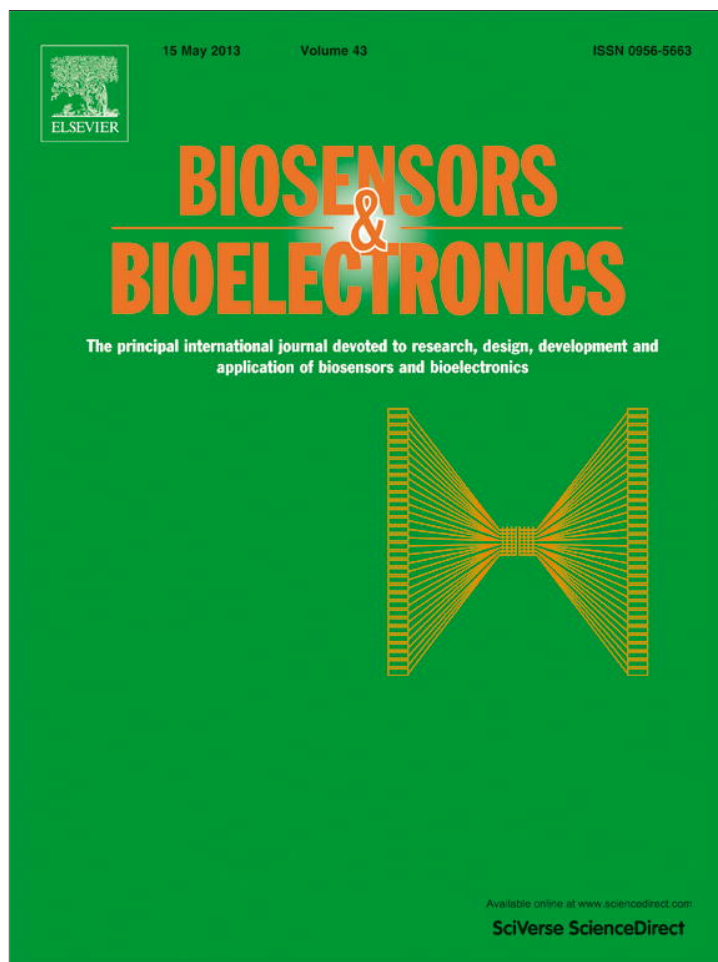


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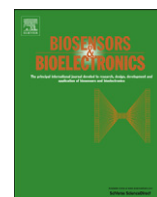
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Multilayer hemin/G-quadruplex wrapped gold nanoparticles as tag for ultrasensitive multiplex immunoassay by chemiluminescence imaging

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ABSTRACT

A multilayer hemin/G-quadruplex DNAzyme wrapped gold nanoparticle (M-DNAzyme/AuNP) tag was designed for ultrasensitive chemiluminescence (CL) imaging. By combining with a disposable protein array, an ultrasensitive and high-throughput multiplex CL immunoassay method was proposed for simultaneous detection of four cancer biomarkers. The M-DNAzyme/AuNP tag was prepared by assembling high ratio of alkylthiol-capped signal DNA containing multiple G-quadruplex sequences to biotinylated DNA on AuNPs and then reacting with hemin to form multilayer hemin/G-quadruplex DNAzyme units. It could be bound to the biotinylated secondary antibody of sandwich immunocomplex by biotin–streptavidin conjugation to catalyze a CL reaction on a protein array, which produced strong CL emission. Under optimal conditions, the CL signals could be simultaneously collected by a charge-coupled device for ultrasensitive multiplex CL imaging of cancer biomarkers. Using α -fetoprotein, human chorionic gonadotrophin- β , carcinoma antigen 125, and carcinoembryonic antigen as model analytes, the proposed immunoassay method showed high sensitivities and wide linear ranges in a simple, cheap and high throughput way. The M-DNAzyme/AuNP as a universal signal tag as well as the protein chip could be suitable for mass production for economical, portable and multianalyte assay, showing a promising potential in application to clinic and other relative fields.

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1. Introduction

Accurate and high throughput detection of biomarkers in serum or tissue samples plays an essential role in early screening and diagnosis of diseases (Stoeva et al., 2006; Yu et al., 2006; Wu et al., 2007a, 2007b). Tumor markers exist in blood at trace levels in the absence of a tumor and their levels rise upon the formation of tumor. The extremely low concentrations of most biomarkers during the early stage of diseases and the limited specificity of single marker in cancer diagnosis (Wu et al., 2007a, 2007b) lead to urgent need of the ultrasensitive multiplex immunoassay methods for simultaneously detecting a panel of tumor markers with easy operation, low cost, and small sample consumption.

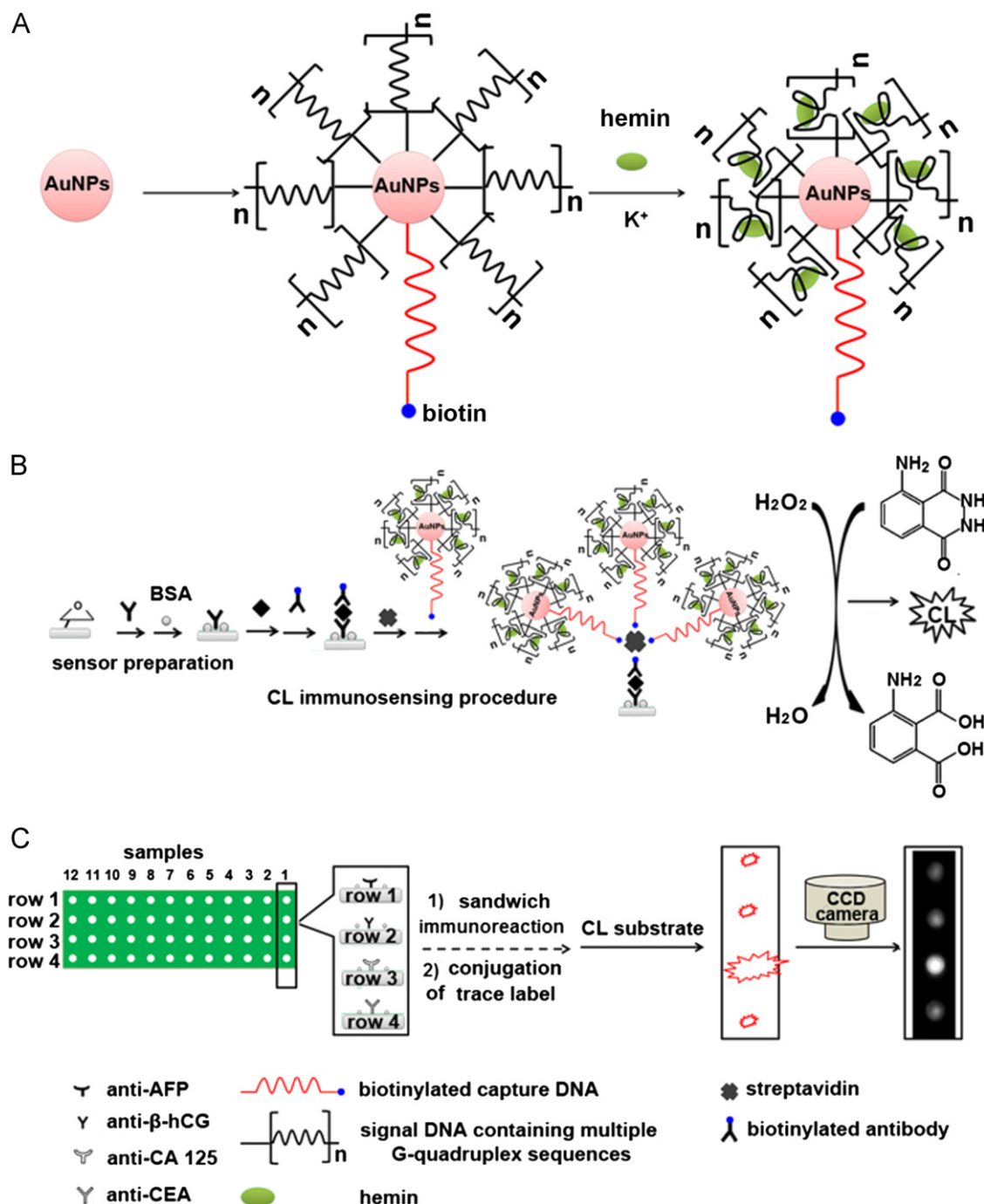
Various protein arrays or immunosensor arrays have been designed for electrochemical (Kojima et al., 2003; Wilson and Nie, 2006a; Wu et al., 2007a, 2007b) and optical (Knecht et al., 2004; Deiss et al., 2009; Hu et al., 2010; Kwon et al., 2011) readout of multiplex analytes. Chemiluminescence (CL) immunosensor array

benefiting from the advantages of simple sensor set-up and without the need of external light source or optics is one of the most developed technologies. In order to improve the sensitivity of CL immunosensing, numerous signal amplification strategies based on various bionanocomposite probes have been designed by loading a large amount of natural enzymes such as horseradish peroxidase (HRP) on nano-carriers (Bi et al., 2009a, 2009b; Zhen et al., 2010). However, the limited species and amount of natural enzymes assembled on the nano-carriers have become a bottleneck. Thus low molecular weight DNAzyme formed by binding a G-quadruplex DNA strand with a hemin molecule has been attempted to be used as a substitute in CL analysis (Niazov et al., 2004; Willner et al., 2008; Wang et al., 2011). As a promising HRP-mimicking enzyme, DNAzyme can catalyze the oxidation of luminol by H₂O₂ to generate CL emission (Xiao et al., 2004). Comparing with the natural enzymes, DNAzyme can be easily produced and possesses better stability and robustness. To further enhance the sensitivity, this work assembled a multilayer structure containing multiple DNAzyme units on the nano-carriers to achieve the signal amplification of CL transduction.

The multilayer DNAzyme structure can generally be prepared using enzyme-assisted DNA replication strategies, such as rolling

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Scheme 1. Schematic diagrams of (A) preparation of M-DNAzyme/AuNP with the optimal n value of 3, (B) sensor preparation and CL immunosensing procedure, and (C) multiplex CL imaging immunoassay of four tumor markers using an immunosensor array.

circle amplification (Cheglakov et al., 2007; Bi et al., 2010), hybridization chain reaction (Shimron et al., 2012) and polymerase chain reaction (Cheglakov et al., 2006). These processes which demand the assistance of primers, polymerase or nicking enzymes are high-cost and complex. Herein, the multilayer structure was prepared by directly assembling high ratio of multiple G-quadruplex sequences to biotinylated DNA on gold nanoparticles (AuNPs), followed by the binding of hemin to the sequences to form multilayer DNAzyme units (Scheme 1A). AuNP can enhance the CL emission of luminol–H₂O₂ system (Zhang et al., 2005) and is one of the most used nano-carriers in CL bioassay (Niazov et al., 2004; Bi et al., 2009a, 2009b). The assembly and formation processes of multilayer DNAzyme

wrapped AuNPs (M-DNAzyme/AuNP) were very simple, and the probe produced a limit of detection down to fM level, which was much lower than the previously reported multilayer DNAzyme strands for CL detection of protein (6.6 pM) (Bi et al., 2010). The presence of biotinylated DNA enabled the multilayer units to be a universal signal tag for CL immunoassay by the specific recognition of biotin to avidin.

By combining the universal tag with a disposable protein immunosensing array and a sensitive charge-coupled device (CCD) detector, an ultrasensitive and high-throughput CL imaging method was proposed for simultaneous immunoassay of multiple biomarkers. Compared to the limit of detection of 4.1 pg mL⁻¹ for CL immunoassay of carcinoembryonic antigen (CEA) using

monolayer DNAzyme functionalized AuNP as label (Wang et al., 2011), the limit of detection of 0.02 pg mL^{-1} indicated a greatly improved sensitivity due to the high loading of DNAzyme units. The proposed method is economical and portable and shows excellent performance of the universal M-DNAzyme/AuNP tag.

2. Materials and methods

2.1. Materials and reagents

Capture antibodies (Ab1) of α -fetoprotein (AFP) (mouse monoclonal antibodies, clone No. bsm-1021), human chorionic gonadotropin- β (β -hCG) (rabbit polyclonal antibodies, clone No. bs-0953R), carcinoma antigen 125 (CA 125) (rabbit polyclonal antibodies, clone No. bs-0091R) and CEA (mouse monoclonal antibodies, clone No. bsm-1023) were all purchased from Beijing Biosynthesis Biotechnology Co. Ltd. (China). The biotinylated detection antibodies (Ab2) of AFP and CA 125 were obtained from CanAg Diagnostics AB (Gothenburg, Sweden), and the Ab2 of β -hCG and CEA were obtained from Beijing Keybiotech Co. Ltd. (China), respectively. The standard solutions of AFP, CA 125 and CEA, as well as the CL substrate solutions for HRP (luminol-*p*-iodophenol and H_2O_2), were from commercial AFP, CA 125 and CEA CL ELISA kits, respectively, which were supplied by Autobio Diagnostics Co., Ltd. (China). Standard solution of β -hCG was from commercial β -hCG CL ELISA kit supplied by Tigsun Diagnostics Co. Ltd. (China). The electrochemiluminescent (ECL) immunoassay reagent kits for reference detection were provided by Roche Diagnostics GmbH (Germany). Streptavidin was obtained from Promega (USA). Chloroauric acid ($\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$) and trisodium citrate were obtained from Shanghai Reagent Company (China). 3-Glycidoxypropyltrimethoxysilane (GPTMS), bovine serum albumin (BSA), and hemin, were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Hemin stock solution ($100 \text{ }\mu\text{M}$) was prepared in 0.01 M pH 9.0 phosphate buffered saline (PBS) and stored in the dark at $4 \text{ }^\circ\text{C}$. Capture DNA ($5'$ -SH-TTT TTT GGT TTG GTC GGT ATG GTT TTT TTG GTT TGG TCG GTA TGG TTT TTT TGG TTT GGT CCG TAT GGT TTT TTT GGT TTG GTC GGT ATG GT-biotin- $3'$) and signal DNA ($5'$ -SH-[TTT TTT GGG TTG GGC GGG ATG GG] $_n$ - $3'$, $n=1, 2, 3$ or 4) were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (China). Tween-20 was obtained from Sinopharm Chemical Reagent Co. Ltd. (China). 0.01 M pH 7.4 PBS was used as coupling buffer. Washing buffer and blocking buffer were 0.01 M pH 7.4 PBS containing 0.05% Tween-20 and 5% BSA, respectively (Zong et al., 2012). Ultrapure water from a Millipore water purification system ($\geq 18 \text{ M}\Omega$, Milli-Q, Millipore) was used in the whole assay. Clinical serum samples were from Jiangsu Cancer Hospital. All other reagents were of analytical grade and used as received.

2.2. Apparatus

Cooled low-light CCD with high resolution (BioImaging Systems Chemi HR 410 camera, UVP, USA) was used to collect the CL signals of the protein array. The kinetic behavior of the CL reaction catalyzed by M-DNAzyme/AuNP tag was studied with a static method by IFFM-E luminescent analyzer (Remax, China). The ultraviolet-visible (UV–vis) absorption spectra were recorded with a Nanodrop-2000C UV–vis spectrophotometer (Nanodrop, USA). The control levels of the tumor markers in sera were obtained with an automated ECL analyzer (Elecys 2010, Roche).

2.3. Preparation of M-DNAzyme/AuNP tag

AuNPs with 13 nm diameter were prepared according to the previous protocol (Ambrosi et al., 2008). Briefly, $40 \text{ }\mu\text{L}$ of $1 \text{ }\mu\text{M}$

biotinylated DNA and $24 \text{ }\mu\text{L}$ of $100 \text{ }\mu\text{M}$ signal DNA were added into 1.0 mL of 4.0 nM AuNP solution and incubated for 16 h with stirring at room temperature. Afterward, 0.01 M pH 7.4 PBS containing 2.0 M NaCl was added stepwise to the mixture to reach a final NaCl concentration of 0.1 M . Then, 0.1 mL of 0.01 M PBS containing 0.1 M KCl was added to the solution and incubated with stirring for 2 h. Subsequently, the unassembled DNA was removed by centrifugation ($10,000 \text{ rpm}$, 30 min, $4 \text{ }^\circ\text{C}$) and the precipitate was redispersed in 0.01 M PBS containing 0.1 M KCl. Excess amount of hemin was added to the suspension and incubated for 1.5 h in dark at $4 \text{ }^\circ\text{C}$ to form hemin/G-quadruplex DNAzyme, and the redundant hemin was removed by centrifugation ($10,000 \text{ rpm}$, 30 min, $4 \text{ }^\circ\text{C}$). The obtained M-DNAzyme/AuNP tag was redispersed in 1.0 mL of 0.01 M pH 7.4 PBS containing 0.1 M KCl to obtain a final concentration of 4.0 nM and kept in dark at $4 \text{ }^\circ\text{C}$ before use.

2.4. Fabrication of disposable protein array

The details for fabrication of the disposable protein array have been previously described (Zong et al., 2012). Briefly, regular microscope glass slides were activated with piranha solution ($\text{H}_2\text{SO}_4/30\% \text{ H}_2\text{O}_2$, 7:3 in volume) for 12 h and silanized with 1% GPTMS/toluene solution overnight at room temperature to bring epoxy groups on their surfaces. Then, a layer of hydrophobic photo-inactive film with 48 cells (2 mm diameter, 4 mm edge-to-edge separation) in a 4×12 format was printed on the silanized glass slide by screen printing technology. $1.5 \text{ }\mu\text{L}$ of Ab1 of AFP, β -hCG, CA 125, and CEA at $10 \text{ }\mu\text{g mL}^{-1}$ was dropped on each cell in rows 1–4, respectively, and incubated overnight at $4 \text{ }^\circ\text{C}$. After washing with washing buffer and drying, $1.5 \text{ }\mu\text{L}$ of blocking buffer was dropped in each cell for 1 h to block the unreacted epoxy group. Upon a washing process, the disposable protein array for four tumor markers was obtained and stored in 0.01 M pH 7.4 PBS at $4 \text{ }^\circ\text{C}$ before use.

2.5. Multiplex CL imaging immunoassay

The CL imaging immunoassay was illustrated in Schemes 1B and C. For CL immunosensing, firstly, $1.5 \text{ }\mu\text{L}$ of the standard solutions of AFP, β -hCG, CA 125, and CEA with different concentrations and their corresponding Ab2 at $2 \text{ }\mu\text{g mL}^{-1}$ were sequentially incubated in the immunosensing cells in rows 1–4 for 15 min, respectively. After rinsing with washing buffer and drying, $1.5 \text{ }\mu\text{L}$ of $2 \text{ }\mu\text{g mL}^{-1}$ streptavidin and $1.5 \text{ }\mu\text{L}$ of the M-DNAzyme/AuNP tag (4.0 nM) were sequentially dropped on all sensing cells to incubate with the sandwich immunocomplexes for 15 and 30 min, respectively, followed by washing and drying. Finally, $1.5 \text{ }\mu\text{L}$ of CL substrates were delivered into the cells to collect the CL signals by a CCD with three 1-min exposure times for dynamic integration of 3 min. To perform the detection of the four biomarkers in one serum sample, $4 \times 1.5 \text{ }\mu\text{L}$ of serum sample were added to four immunosensing cells in one column to perform the same procedures. The spots obtained by the CCD collection were automatically identified using VisionWorks®LS image acquisition and analysis software (UVP, USA). The CL intensity of each spot was calculated as the mean pixel intensity within a square of a given side length around each spot center.

3. Results and discussion

3.1. Optimization of the synthesis conditions of M-DNAzyme/AuNP tag

The proposed M-DNAzyme/AuNP tag employed signal DNA to obtain multilayer DNAzyme strands for catalyzing CL reaction and

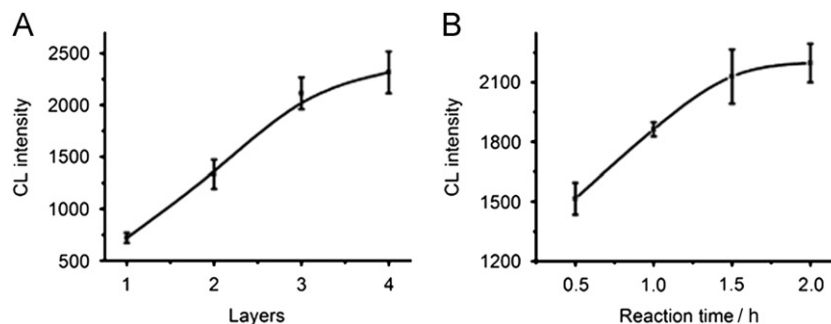


Fig. 1. Effects of (A) layer number of G-quadruplex, and (B) reaction time between hemin and G-quadruplex for preparation of M-DNAzyme/AuNPs. 0.01 ng mL⁻¹ CEA was used for the optimization.

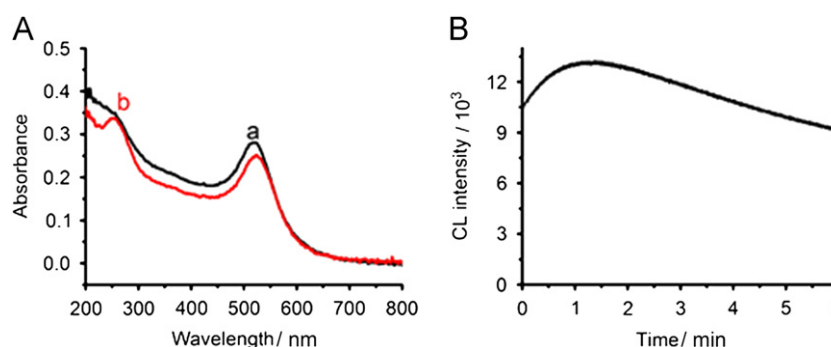


Fig. 2. (A) UV-vis spectra of AuNPs (a) and M-DNAzyme/AuNPs (b), and (B) kinetic curve of the CL reaction catalyzed by M-DNAzyme/AuNP tag.

biotinylated DNA to bind the sandwich immunocomplexes. The ratio of signal DNA to biotinylated DNA was firstly optimized to be 60:1. The layer number of G-quadruplex sequences in signal DNA was then optimized at this ratio. As shown in Fig. 1A, the intensity of CL emission underwent a multiplicative increase with the increasing layer number until to layer 3, indicating a multiplicative increase of DNAzyme units from layer 1 to 3 with a negligible steric hindrance of the DNA strand. However, when the layer number was higher than 3, the increase of CL intensity became slower, which was due to the bigger steric hindrance of the longer DNA strand. Thus, a signal DNA containing three layers of G-quadruplex sequences was used for the preparation of M-DNAzyme/AuNP tag.

The effect of the reaction time between hemin and G-quadruplex sequences in signal DNA was shown in Fig. 1B. The CL emission increased with the increasing reaction time, and trended to its maximum value at 1.5 h, indicating that almost all the G-quadruplex structures on AuNPs were bound to form DNAzyme units. Thus, a reaction time of 1.5 h was chosen.

3.2. Characterization and CL reaction kinetic of M-DNAzyme/AuNP tag

Fig. 2A shows the UV-vis spectra of AuNPs and M-DNAzyme/AuNPs. The size of the AuNPs could be estimated to be 13 nm from its absorption peak at 519 nm (curve a), which was further confirmed by TEM. Compared with the AuNPs, the UV-vis spectrum of M-DNAzyme/AuNPs showed a red shift of the absorption peak to 523 nm and another strong absorption at approximately 260 nm due to the adsorption of the guanine-rich DNA strand (curve b), indicating the successful loading of DNAzyme on the AuNPs. By determining the CL signal catalyzed by M-DNAzyme/AuNP tag and quantifying the value in a calibration curve obtained from free DNAzyme under the same CL catalytic conditions, the number of active DNAzyme units in per probe was estimated to be 160, which was much higher than that of 10 for

the HRP-antibody/AuNP label (Ambrosi et al., 2008). Although each M-DNAzyme/AuNP tag contained lower total amount of DNAzyme than HRP on each magnetic bead with a diameter 76 times larger than AuNPs (Mani et al., 2009), the surface enzyme coverage was 126 times higher than that on the magnetic bead, which would bring high detection sensitivity and wide linear range for the proposed CL immunoassay.

The kinetic behavior of the CL reaction catalyzed by the newly designed M-DNAzyme/AuNP tag was studied in a static format. As seen from Fig. 2B, the CL intensity corresponding to the reaction catalyzed by M-DNAzyme/AuNP tag increased quickly and reached the maximum value within 1 min. Afterwards the CL intensity decreased slowly, and could retain 80% of the maximum value after 3 min. In order to obtain high detection sensitivity, the CCD detector normally demanded a long exposure time for CL signal collection. According to the kinetic behavior of M-DNAzyme/AuNP catalyzed CL reaction, an exposure time of 3 min was chosen for dynamic integration of CCD to collect the CL signal.

3.3. Signal amplification by M-DNAzyme/AuNP tag

In order to evaluate the amplification ability of M-DNAzyme/AuNP tag, sandwich immunoassays of 1.0 ng mL⁻¹ CEA using both the tag and HRP/AuNP labeled antibody (Zong et al., 2012) were performed on the same protein array. As shown in Fig. 3, the M-DNAzyme/AuNP tag showed the CL intensity of 3914 and 14015, while the latter produced the CL intensity of 909 and 2689 at the exposure times of 3 and 10 min, respectively. Obviously, the designed tag produced stronger CL signal. Thus this work could use a 3-times shorter exposure time than the previous work (Zong et al., 2012), leading to higher detection throughput.

3.4. Analytical performance

With a sandwich immunoreaction and two steps of biotin-streptavidin reaction, M-DNAzyme/AuNP tag was bound to the

sandwich immunocomplex to trigger the CL reaction. Under optimal conditions, the brightness of the collected spots on the protein array increased with the increasing concentrations of analytes (Fig. 4a). The CL intensity was proportional to the logarithm value of analyte concentration over the ranges of 1.0×10^{-3} – 100 ng mL^{-1} for AFP, 2.0×10^{-4} – 20 IU L^{-1} for β -hCG, 1.0×10^{-4} – 10 U mL^{-1} for CA 125, and 1.0×10^{-4} – 10 ng mL^{-1}

for CEA, respectively (Fig. 4b). The limits of detection corresponding to the signals of 3SD for the 4 biomarkers were $2.7 \times 10^{-5} \text{ ng mL}^{-1}$, $1.1 \times 10^{-5} \text{ IU L}^{-1}$, $1.7 \times 10^{-5} \text{ U mL}^{-1}$ and $2.0 \times 10^{-5} \text{ ng mL}^{-1}$, respectively, which were much lower than those reported previously in DNAzyme-based immunoassay (Zhou et al., 2009; Wang et al., 2011), CL immunoassays with other amplification strategies (Bi et al., 2009a, 2009b; Wu et al., 2009) and multiplexed immunoassays (Wilson and Nie, 2006b; Yang et al., 2009; Ge et al., 2012; Wang et al., 2012). The sensitivities as slopes of the calibration curves in Fig. 4b for AFP, β -hCG, CA 125, and CEA were 933.6 mL ng^{-1} , 905.9 LIU^{-1} , 799.9 mL U^{-1} , and 830.8 mL ng^{-1} , respectively, which were much larger than those of the previous work using HRP/AuNP labeled antibody as label (427.9 mL ng^{-1} for AFP, 508.8 mL U^{-1} for CA 125, and 534.1 mL ng^{-1} for CEA) (Zong et al., 2012). High sensitivities and low limits of detection could improve the detection precision and be suitable for detecting low-abundance biomarkers in serum samples. More importantly, the proposed CL imaging immunoassay method showed a wide detection range of five orders of magnitude for all the four biomarkers, indicating great promise for practical application. The M-DNAzyme/AuNP tag as well as the protein array could be stored at 4°C in dark. After the tag or protein array was stored for 15 days, the CL signals of the AFP, β -hCG, CA 125 and CEA could remain more than 90% of their initial intensities, indicating acceptable stability.

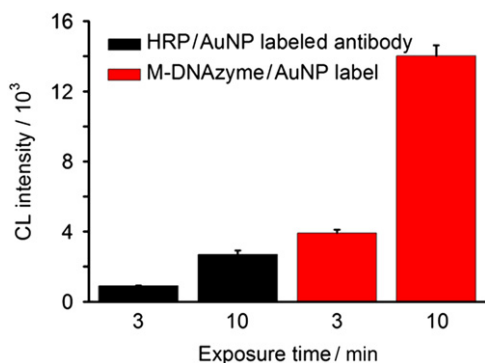


Fig. 3. Performance of M-DNAzyme/AuNP tag compared with HRP/AuNP labeled antibody for CL immunoassay of 1.0 ng mL^{-1} CEA under optimal detection conditions.

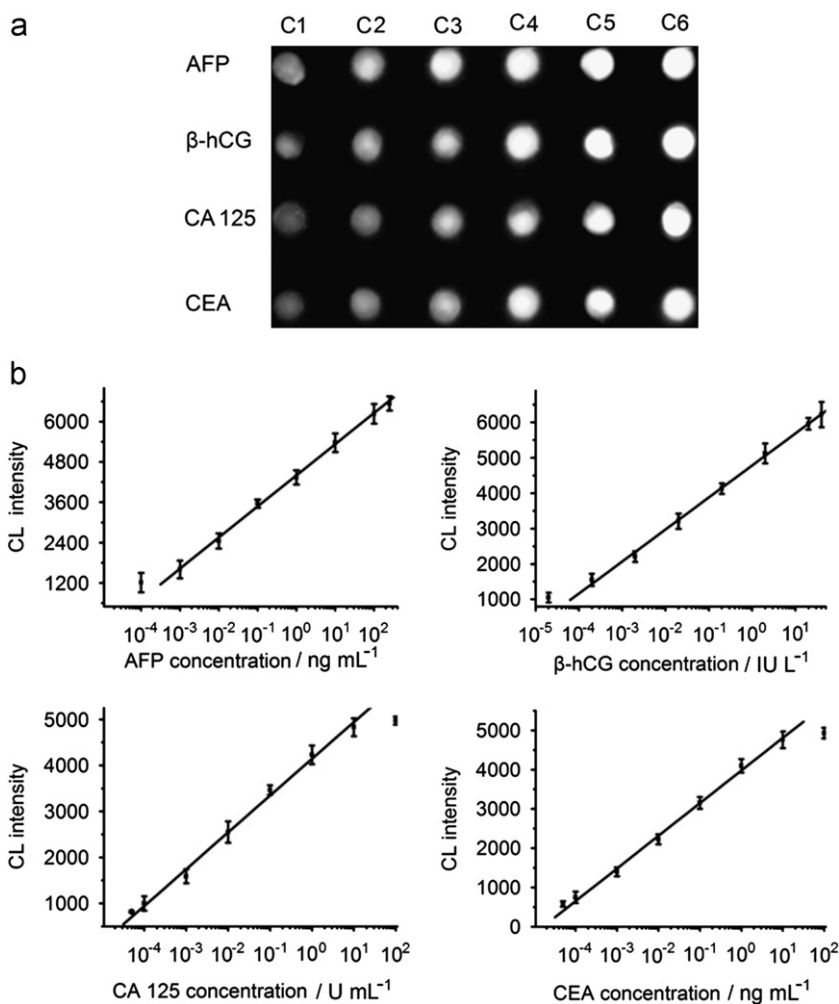


Fig. 4. CL image (a) and calibration curves (b) for immunoassay of four biomarkers at 1.0×10^{-3} , 0.01, 0.1, 1.0, 10 and 100 ng mL^{-1} AFP, 2.0×10^{-4} , 2.0×10^{-3} , 0.02, 0.2, 2.0 and 20 IU L^{-1} β -hCG, 1.0×10^{-4} , 1.0×10^{-3} , 0.01, 0.1, 1.0 and 10 U mL^{-1} CA 125, and 1.0×10^{-4} , 1.0×10^{-3} , 0.01, 0.1, 1.0 and 10 ng mL^{-1} CEA from C1 to C6, respectively.

Table 1

Assay results of clinical serum samples using the proposed and reference methods.

Biomarkers	Sample no.	1	2 ^a	3	4 ^a	5 ^b	6 ^c
AFP (ng mL ⁻¹)	Proposed method	3.60	2.33	1.53	2.24	3.16	3.66
	Reference method	3.72	2.28	1.62	2.21	3.20	3.41
	Relative error (%)	-3.2	2.2	-5.6	1.4	-1.3	7.3
β -hCG (IU L ⁻¹)	Proposed method	2.03	0.11	1.04	0.58	4.43	2.50
	Reference method	2.14	0.12	1.07	0.53	4.63	2.49
	Relative error (%)	-5.1	-8.3	-2.8	9.4	-4.3	0.4
CA 125 (U mL ⁻¹)	Proposed method	10.14	12.70	5.56	23.65	246.9	3126
	Reference method	9.59	12.40	6.05	22.76	253.7	2983
	Relative error (%)	5.7	2.4	-8.1	3.9	-2.7	4.8
CEA (ng mL ⁻¹)	Proposed method	1.64	1.00	3.13	1.74	1.58	1.27
	Reference method	1.69	0.96	3.06	1.58	1.50	1.29
	Relative error (%)	-3.0	4.2	2.3	10.1	5.3	-1.6

^a The serum samples were diluted at 10 times, respectively.^b The serum samples were diluted at 100 times, respectively.^c The serum samples were diluted at 1000 times, respectively.

3.5. Sample throughput

High sample throughput of protein detection required short analytical time for per assay. In this work, the total testing process of the 48 detections in the 4×12 array could be completed within 85 min, including 30 min for two-step sandwich immunoreaction, 45 min for conjugation of the tag to immunocomplexes by biotin-streptavidin reaction, 3 min for signal collection, and several minutes for washing and drying, and this led to a throughput of 34 tests per hour for single analyte measurement and eight samples per hour for detecting a panel of four tumor markers in every sample. When parallel incubation was performed conveniently on numerous arrays, the collection time of only 3 min for each array could greatly increase the detection throughput.

3.6. Evaluation of cross-reactivity and cross-talk

The cross-reactivity between analytes and nonspecific antibodies was investigated. Using CEA immunosensing cells on the protein array as model, the cross-reactivity was evaluated by comparing the CL intensity after incubation with blank solution, the mixture of 1.0 ng mL^{-1} AFP, 1.0 IU L^{-1} β -hCG and 1.0 U mL^{-1} CA 125, 1.0 ng mL^{-1} target CEA and the mixture of CEA and other antigens. As expected, the CEA immunosensing cells showed obvious responses to target CEA and the mixture containing target antigen, while negligible response was observed in the absence of target CEA (Supplementary Fig. S1), indicating negligible cross-reactivity and nonspecific binding. In addition, as the protein array was used to detect the corresponding target individually, the CL signals occurred only in corresponding cell. Therefore, no cross-talk occurred among neighboring immunosensing cells.

3.7. Detection of cancer biomarkers in serum samples

To evaluate the analytical reliability and application potential of the proposed multiplex CL imaging immunoassay method, the assay results of four cancer biomarkers in human serum samples from cancer patients obtained by the designed protein array were compared with the reference values obtained by commercial electrochemiluminescent single-analyte testing. When the levels of cancer biomarkers were over their calibration ranges, serum samples were appropriately diluted with 0.01 M pH 7.4 PBS prior to assay. The results with relative errors less than 10.1% for the detection of all the four tumor markers (Table 1) were acceptable, indicating the practicability of the proposed method and the multilayer DNAzyme tag.

4. Conclusions

This work designed a new multilayer DNAzyme wrapped AuNP tag to combine with a disposable protein array for CL imaging immunoassay of multiple biomarkers. The formation of multilayer DNAzyme strands on AuNPs was very simple and low-cost (\$7 per array). The high surface coverage of DNAzyme on the label led to ultrahigh sensitivity and short collection time of CL signals. The M-DNAzyme/AuNP tag could easily be linked to the sandwich immunocomplex by biotin-streptavidin conjugation, and serve as a universal signal label for multiple analytes. Using CCD to collect the CL signals triggered by M-DNAzyme/AuNP tag, the proposed method could simultaneously detect four biomarkers in 12 samples with a protein array. Although the immunoassay process contained a sandwich immunoreaction and two steps of biotin-streptavidin reaction, the short signal collection time led to a high detection throughput when parallel incubation was performed on several arrays. The proposed method and M-DNAzyme/AuNP tag exhibited high sensitivity, wide linear range and acceptable accuracy, showing promising application in early cancer diagnosis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2012.12.051>.

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