Chemiluminescence DNA biosensor based on dual-amplification of thrombin and thiocyanuric acid-gold nanoparticle network†

Xuemei Li, Wei Li and Shusheng Zhang*

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A sensitive DNA biosensor based on dual-amplification of thrombin and thiocyanuric acid-gold nanoparticle (TCA-AuNP) network is developed. First, the sandwich hybridization is formed by the capture probe immobilized on the surface of magnetic beads (MBs), the target DNA and the reporter probe loaded on PbS nanoparticles (PbS NPs). The PbS NPs contain two kinds of DNA sequences, one is the reporter probe complementary to the target DNA, and the other is the thrombin aptamer I. Through the specific recognition for thrombin, thrombin aptamer II labeled gold nanoparticles are linked to the sandwich complex, and further fabricate a network with TCA. AuNPs are released and dissolved into Au^{3+}, which catalyzes luminol chemiluminescence (CL) reaction. Due to the dual-amplification effects of thrombin-labeled PbS NPs and the TCA-AuNP network, a significant sensitivity enhancement of this DNA biosensor could be obtained, in the range of 2.0×10^{-16} M to 3.5×10^{-14} M, with a limit of detection (LOD) as low as 1.0×10^{-16} M.

Introduction

Developing sensitive, reliable, and low-cost sensing methods to allow sequence-specific detection of DNA targets associated with either genetic or pathogenic diseases has become increasingly important in early disease screening and molecular diagnostics. Great efforts have been made toward enhancement of detection sensitivity by signal amplification.[1–3] Polymerase chain reaction (PCR), an enzyme-based DNA amplification technology, is often employed toward these applications.[4] However, PCR is often criticized for its complex, expensive, time-consuming, and labor-intensive procedure and narrow target DNA quantification range after PCR amplification.[5] Previous reports have involved enzymes as amplifiers that can translate a single hybridization event into over 10 000 enzyme turnovers.[6,7] However, commercialization of these sensors might be hampered by either high cost or relatively poor stability of enzymes.

Nanoparticles can also serve as redox labels for DNA and protein detection, taking advantage of their high stability, low cost, and labeling convenience.[8,9] Two types of detection strategies have been adopted. In the first method, the nanoparticles were used as labels and oxidatively dissolved using a strong oxidant, and then electrochemical or CL detection method was used to detect the dissolved metallic ions.[10–12] The other popular method was using nanoparticles as carriers to load a large amount of active species or bio-bar-code DNA.[13–16] Mirkin *et al.* reported a bio-bar-code modified gold nanoparticle (AuNP) method for nucleic acid and protein detection, which was shown to be as sensitive as PCR.[17–19] Recently, our group reported electrochemical and CL detection strategies using DNA-labeled AuNPs as carriers for $[Ru(NH_3)_6]^{3+}$ and CuS nanoparticles.[20–22]

Aptamers are nucleic acid ligands that bind with high affinity to their target molecules and have been shown to be particularly useful for the detection of protein analytes.[23] In comparison to antibodies, aptamers possess certain advantages, containing high selectivity, stability, target versatility, and convenient regeneration and modification. Aptamers offer excellent prospects for protein detection because of their high selectivity and affinity toward their targets. In a reported approach,[24] aptamer-functionalized Pt NPs were employed as catalytic labels. The Pt NPs catalyzed the electrochemical reduction of H_2O_2 and amplified detection of thrombin with a LOD of nM.

In the present work, taking advantages of the specific interaction between thrombin and a pair of aptamers that bind to different regions of thrombin, a sensitive DNA biosensor was fabricated. Due to the dual-amplification effects of thrombin-labeled PbS NPs and the TCA-AuNP network, sensitive detection of specific nucleic acids sequences on the basis of the hybridization reaction can be improved greatly.

Experimental

Chemicals

All oligonucletides used in the present study were purchased from SBS Genetech Co., Ltd. (Beijing, China), and their sequences were as follows: capture probe (S_1): 5′-NH_2-$(CH_2)_6$-GGG-TCT-GAG-GGA-3′; target sequence (S_2): 5′-TCC-CTC-AGA-CCC-AAT-GTG-CTC-CCC-CAA-CTC-CTC-3′; probe complementary to target DNA (S_3): 5′-GAG-GAG- TTG-GGG-GAG-CAC-ATT-$(CH_2)_6$-NH_2-3′; thrombin aptamer I (S_4): 5′-NH_2-$(CH_2)_6$-GGT-TGG-TGT-GGT-TGG-3′; thrombin aptamer II (S_5): 5′-HS-$(CH_2)_6$-AGT-CCG-TGG-TAG-GGC-AGG-TTG-GGG-TGA-CT-3′.

Key Laboratory of Eco-chemical Engineering, Ministry of Education, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao, 266042, China. E-mail: shushzhang@126.com.; Fax: +86 532-84022750

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1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), thrombin and bovine serum albumin (BSA) were from Sigma (St. Louis, MO), and used without further purification. Thiocyanuric acid (TCA) and tri(2-carboxyethyl)phosphine hydrochloride (TCEP, 98%) were purchased from Alfa Aesar (MA, USA). A luminol (standard powder, Sigma-Aldrich) stock solution (2.5×10^{-2} M) was prepared by dissolution in 1.0 M NaOH and stored in the dark. The stock solution was consecutively diluted with 0.1 M NaOH/$NaHCO_3$ to the proper solution used for CL determination. Other chemicals employed were all of analytical grade and double distilled water (DDW) was used throughout. MBs modified with carboxyl groups (0.5–1.0 μm, 10 mg mL^{-1}) and magnetic rack were obtained from BaseLine Chrom Tech Research Centre (Tianjin, China).

Apparatus

The CL measurements were performed with a BPCL ultraweak luminescence analyzer (Institute of Biophysics Academic Sinica, Beijing, China). Transmission electron microscopy (TEM) was taken with JEM-2000EX/ASID2 instrument (HITACHI, Japan). UV-visible spectra were carried out on a Cary 50 UV-vis spectrophotometer (Varian, USA).

Preparation of AuNPs and labeling with thrombin aptamer II

Gold nanoparticles were prepared by citrate reduction of $HAuCl_4$ with trisodium citrate according to the literature.[25] Briefly, appropriate amounts of 38.8 mM sodium citrate was immediately added to 1.0 mM $HAuCl_4$ (100 mL) refluxing solution under stirring, and the mixture was kept boiling for another 15 min. The solution color turned from yellow to a wine red, indicating the formation of different sizes of AuNPs, and was cooled to room temperature with continuous stirring.

The mercapto aptamer II (45.7 pM) was activated with acetate buffer (pH 5.2) and 10 mM TCEP (1.5 μL) for 1 h, and then added to 1 mL of freshly prepared AuNPs and shaken gently overnight. Over the course of 16 h, the DNA-AuNP conjugates were aged in salts (0.1 M NaCl, 10 mM acetate buffer) for another 24 h. Excess reagents were removed by centrifuging at 15 000 rpm for 15 min. The red precipitate was washed, recentrifuged, and dispersed in 1 mL of buffer containing 300 mM NaCl, 25 mM tris(hydroxymethyl)aminomethane (Tris) acetate, pH 8.2.

Preparation of PbS NPs and modified with S_3 and S_4-thrombin conjugate

Carboxyl modified lead sulfide nanoparticles were prepared according to the literature.[26] Mercaptoacetic acid (9.2 μL) was added to 0.4 mM of $Pb(NO_3)_2$ (50 mL) solution under vigorous stirring, and the pH was adjusted to 7.0 with 0.5 M NaOH. The solution was bubbled with nitrogen for 30 min, and 38.5 mL of 1.3 mM Na_2S was added dropwise to the solution. The reaction was kept for 24 h under bubbling nitrogen until the color turned from colorless to brown.

The process of DNA and thrombin labeling was performed as follows.[14] The mixture of 1.5 pM of S_3 and 44.8 pM S_4 was added into a 200 μL of 0.1 M imidazole solution (pH 6.8). After 30 min, 0.1 M EDC solution (100 μL) and 5.0 mL of PbS colloid (adjusted to pH 3.5 with 0.1 M HCl) were added to the mixture and incubated at room temperature for 12 h with gentle shaking. Excess reagents were removed by centrifugation at 10 000 rpm for 30 min. The precipitate was washed and then dispersed into water. The solution of DNA modified PbS NPs was stored at 4 °C for further use. To the modified PbS nanoparticles, a definite concentration of thrombin in 0.1 M PBS buffer was added. After incubation for 30 min at 37 °C, excess thrombin was removed by centrifugation at 10 000 rpm for 30 min.

Preparation of capture probe-immobilized MBs

The capture probe was immobilized onto the MB through the amidation between amino probe and carboxylic group coated on the MB surface. A suspension of carboxyl MBs (5 μL) was washed three times with 0.1 M imidazol–HCl buffer (pH 6.0, 100 μL). A 0.2 M EDC solution (30 μL) was added to the MB solution and the mixture was incubated at room temperature for 30 min to activate the carboxylate groups on the MBs. Then, 1.5 pM amino capture probe was added and the resulting mixture was stirred for 1 h. After magnetic separation, MBs were washed three times with buffer A (7 mM Tris-HCl, pH 8.0, 0.5 M NaCl) and resuspended in 20 μL buffer A containing 10% BSA for 1 h to eliminate the risk of unspecific binding. The conjugates were resuspended in 20 μL of buffer A before use.

Fabrication of hybridization assay and signal amplification

For the fabrication of DNA sandwich format, MB modified with capture probe was hybridized with 30 μL of different concentrations of target DNA sequences for a desired time at 37 °C. After magnetic separation, 10 μL of the prepared PbS NPs modified with S_3 and S_4–thrombin conjugate, aptamer II labeled AuNPs and an appropriate amount of TCA were added, and the final volume was adjusted to 100 μL with 0.1 M PBS buffer containing 1.0 mM Mg^{2+} (pH 7.3). After incubation for 30 min at 37 °C, the conjugates were washed with 100 μL PBS buffer and the supernatant was removed through the magnetic separation. For comparison, no amplification was also measured without the addition of TCA.

CL measurements

The mixture was resuspended in 50 μL of freshly prepared Au-NP buffer (0.15 M NaCl, 0.01 M sodium phosphate, 0.1% sodium dodecyl sulfate SDS, pH 7.4) containing 0.1 M DTT to release the AuNPs from the thiolated oligonucleotides and TCA network. Magnetic particles were removed from the mixture using the magnetic separator. A solution of 0.25 mM Br_2–0.01 M HCl–0.1 M NaCl (1 mL) was added to the supernatant and reacted for 10 min. The resulting mixture was placed in the oven at 60 °C for 1 h to remove any remaining bromine. A 100 μL of the solution was introduced into a quartz cuvette containing 1.0×10^{-4} M luminol in 0.1 M NaOH/$NaHCO_3$, and the CL signal was measured in the luminescence analyzer.

Results and discussion

Experimental principle of formation of the sandwich assay and the signal amplification

The principle of the sandwich assay with thrombin-labeled PbS NPs and TCA–AuNP network amplification was depicted in Scheme 1. Amino capture probe was immobilized on the surface of magnetic beads MBs. The target DNA was first hybridized with the capture probe and then sandwiched by S_3 loaded on the PbS NPs. Next, the conjugates were further mixed with AuNP labeled aptamer II and TCA. Aptamer II bound tightly and specifically to the thrombin, linking AuNPs to the sandwich conjugates. AuNPs interacted with TCA to form thiocyanuric acid-gold nanoparticle network through Au–S bonds,[27] leading to signal amplification.

To detect the colloidal gold by CL in the final step of the hybridization, the gold metal must be released from the immobilized bound fraction. Dithiothreitol (DTT), a common disulfide reducing agent,[28] was used to liberate the covalently attached AuNP from DNA sequences and TCA for assay readout. DTT has been demonstrated to efficiently remove thiolated oligonucleotides from gold surfaces.[28] After magnetic separation, HCl–NaCl–Br_2 solution was added to the supernatant to oxide the AuNP to the soluble ionic Au form, which was proved to be more efficient than the typical HNO_3–HCl solution, and provided an instantaneous gold dissolution.[29] The gold ions ($AuCl_4^-$) thus released in solution were quantitatively determined by the $AuCl_4^-$ catalyzed luminol CL reaction. The CL signal was directly proportional to the amount of target DNA in the standard.

Characterization of the nanoparticles

The AuNPs, PbS NPs and AuNP–PbS NP conjugates were verified by TEM, from which one can see that the average diameters for AuNPs and PbS NPs were 17 nm and 8 nm, respectively. It was further clarified by UV-visible absorption spectra as shown in Fig. 1. Curve e exhibited both the characteristic absorbance of DNA at 260 nm (curve a) and the characteristic absorbance of Au colloid (curve b) at 520 nm. The results indicated that the PbS NP–DNA–AuNP complexes have been generated.

CL detection conditions

The CL measurement for the $AuCl_4^-$ ion in the gold dissolution was achieved with an alkaline luminol solution as substrate to initiate the CL emission. The CL intensity was found to be greatly affected by the sequence of the sample injection. The comparison of different sample injection sequences were investigated as shown in Fig. S1 in the ESI.†According to the results, 1.0×10^{-4} M luminol in 0.1 M NaOH/$NaHCO_3$ was injected for the following experiments.

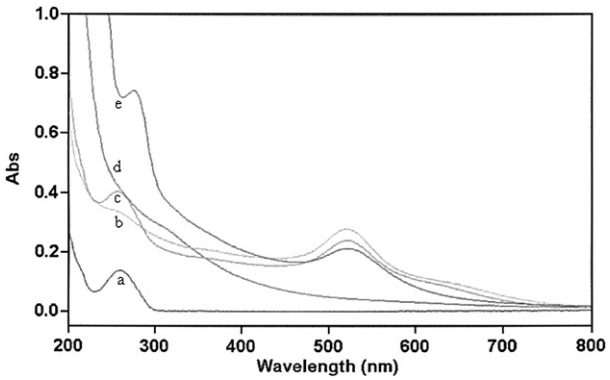
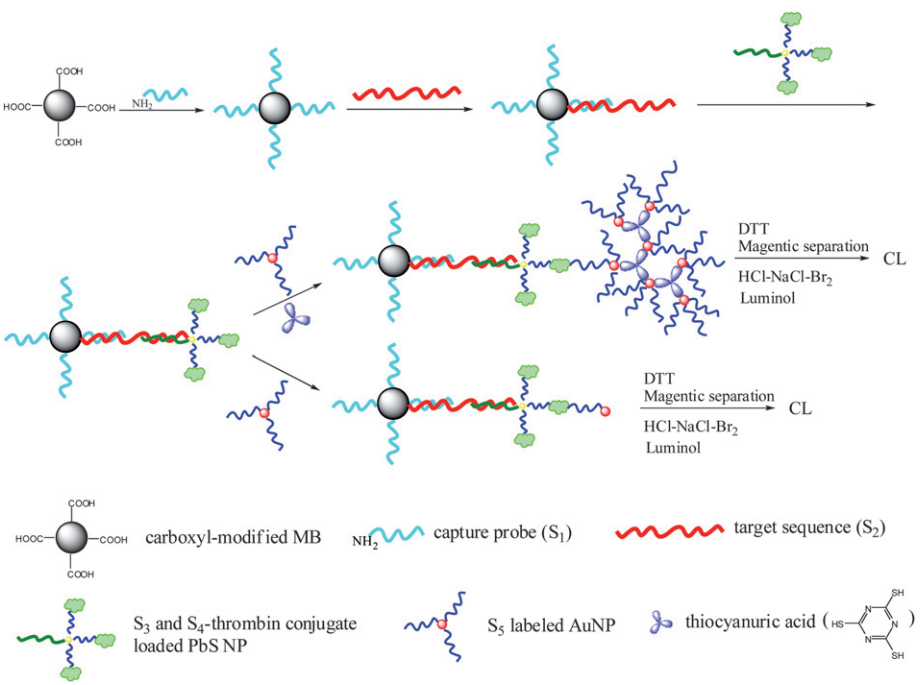


Fig. 1 UV-visible spectra of (a) aptamer I, (b) AuNP, (c) S_5 labeled with AuNP, (d) PbS nanoparticle and (e) PbS NP-DNA-AuNP complex.



Scheme 1 Schematic representation of the sandwich assay and the signal amplification

Effect of the concentration of TCA on CL detection

The concentration of TCA is also an important factor for the CL detection. Solutions of AuNPs and TCA should mix in appropriate amounts. Since optimal TCA concentration provides maximum signal amplification, thus improving sensitivity. Fig. S2, ESI,† showed TCA concentration-dependent CL signals. The effects of different concentrations of TCA on the CL intensities were investigated under two target concentrations (3.0×10^{-10} M and 3.0×10^{-14} M). For both cases, as the concentration of TCA increased, the CL intensity increased up until 7.5 μM. After that, the CL intensity decreased gradually. Thus, the optimal concentration for TCA was 7.5 μM.

Optimum of AuNP size

The effect of the size of AuNPs was investigated. A delicate balance between large loading surfaces to allow more TCA loading and small volume to allow the nanoparticles to remain suspended in solution needs to achieved. Our results showed that the largest signal amplification was observed with the diameter of the AuNPs being 17 nm (see Fig. S3 in the ESI†). These results might be attributed to the fact that larger AuNPs would be aggregated in the solution and smaller ones with decreasing numbers of S_5 sequences available for binding to thrombin molecules.

Sensitive detection of target DNA

To examine the sensitivity of the protocol, the CL intensity of the AuNPs released into the solution was measured after hybridization with the target DNA of different concentrations. Fig. 2 showed an increase of the CL intensity with the increase of target DNA concentration, implying that one could use this DNA sensor to perform quantitative target DNA detection. For comparison, CL measurements of AuNPs without TCA with the same target concentrations were also investigated. The CL intensity observed in the presence of TCA was 3 times higher

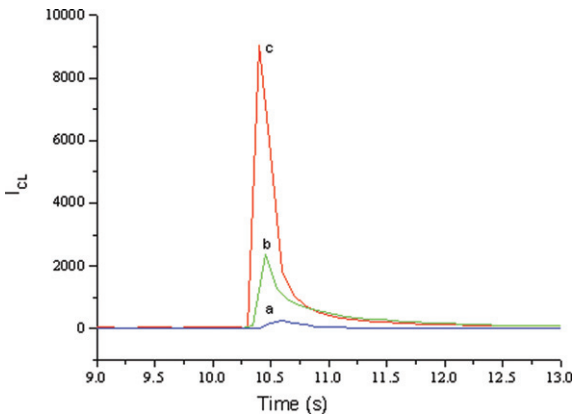


Fig. 2 Kinetic curves of luminol–$AuCl_4^-$ chemiluminescence reactions in the absence of target DNA (a), in the presence of target DNA without TCA enhancement (b), and enhanced by TCA (c). Conditions: luminol, 1.0×10^{-4} M in 0.1 M NaOH/$NaHCO_3$ solution, the concentration for target DNA was of 3.0×10^{-10} M.

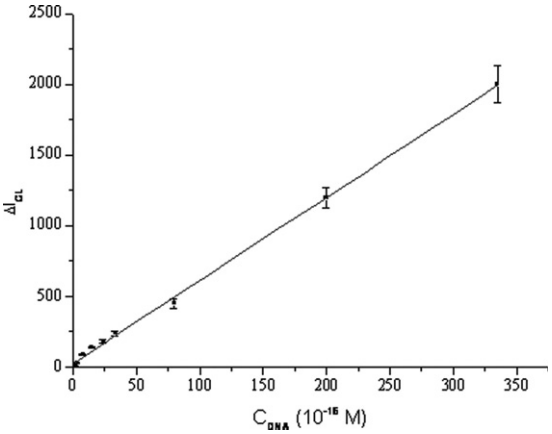


Fig. 3 The calibration curve for the determination of target DNA with TCA amplification.

than that without TCA. We ascribed the enhancement of the signal to the fact that TCA can easily cap more gold nanoparticles to form network-like thiocyanuric acid-gold nanoparticles by the strong interaction among gold nanoparticles and three thiol groups of each thiocyanuric acid molecule.[30] Since Pb^{2+} is also a good catalyst for luminol CL, the solution was further measured using anodic stripping voltammetry (ASV) to test whether some Pb^{2+} from PbS nanoparticles existed in the solution. Pb^{2+} displayed ASV peak at −0.46 V, while no response was observed for the detection solution (Fig. S4 in the ESI†), from which one can see that there is no Pb^{2+} in the solution.

Fig. 3 represented the relationship between the CL intensity changes and target DNA concentrations. In the absence of TCA, the CL intensities increased flatly with the increase of DNA concentration. However, with the signal amplification, the results showed that CL intensities increased with a larger slope with increase of the concentration of target DNA. The calibration plot exhibited a good linear relationship ranging from 2.0×10^{-16} M to 3.5×10^{-14} M, with a correlation coefficient of 0.9938. A detection limit of 1.0×10^{-16} M target DNA can be estimated using 3σ. The use of network-like TCA–AuNP indicated that the detection limit had a 1000-fold improvement than that without enhancement (Fig. S5 in the ESI†). The present strategy was more sensitive than those of nanoparticle, nanotube, conductive polymer, and other nanostructure modified assays as reported previously.[2,22,31]

Selectivity of the DNA biosensor

To demonstrate the specific aptamer interaction, PbS NPs labeled with S_3 alone were used as control to study the non-specific aptamer recognition. Compared with the use of S_3–PbS NP–S_4 conjugates, the CL intensity of S_3–PbS NP was almost negligible. These results indicated that, in the sandwich assay detection model, a high specificity of thrombin and its aptamers could be achieved.

The selectivity of the present biosensor in discriminating perfect complementary DNA from single base mismatched and

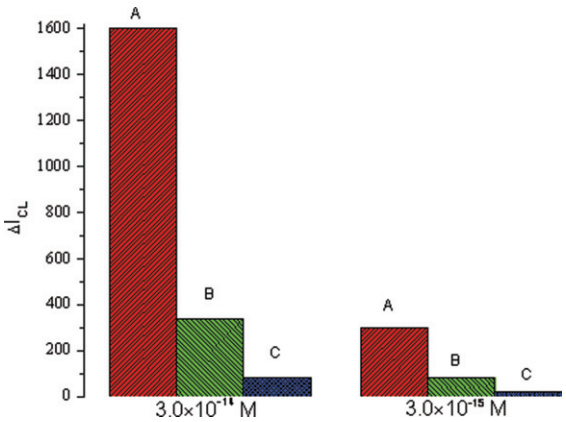


Fig. 4 Comparison of CL signal for the TCA–AuNP network like sensor hybridized with a series of target DNA (3.0×10^{-14} and 3.0×10^{-15} M): the cDNA (A), one-base mismatched DNA (B), and completely mismatched target DNA (C).

non-complementary DNA sequences were investigated under two concentrations as shown in Fig. 4. A well-defined CL signal of luminol–$AuCl_4^-$ was observed for the complementary sequence. The CL intensities for single-base mismatched and non-complementary sequences were significantly weaker than that of the complementary sequence. Referring to the complementary hybridization at each concentration as 100%, the ratio of hybridization efficiencies were as follows: target : Ms : Nc = 100 : 21 : 5 and 100 : 27 : 7 for 3.0×10^{-14} and 3.0×10^{-15} M targets, respectively.

Reproducibility of the DNA biosensor

Besides sensitivity and selectivity, the reproducibility is also one major problem of the DNA biosensor. The reproducibility of the sensor was investigated by repetitive measuring of 3.0×10^{-15} M target DNA in seven individual strategies. From the results shown in Fig. 5, one can see that seven repetitive measurements yielded reproducible CL response with the relative standard deviation of these measurements of 8.2%, showing the good reproducibility of the protocol.

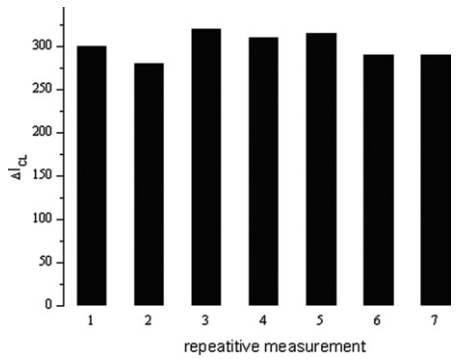


Fig. 5 Results of seven repetitive measures of 3.0×10^{-15} M target DNA.

Conclusions

In conclusion, a novel approach to DNA detection exhibiting excellent sensitivity and selectivity was reported, with the limit of detection for target DNA as low as 1.0×10^{-16} M. The high sensitivity was contributed to the dual-amplification efforts: first, the thrombin labeled on the PbS NPs through the specific interaction with its aptamers; second, TCA–AuNP network introducing more AuNPs capped for each sandwich format. These features make it a promising alternative to conventional single amplification strategy of nanoparticles.

Acknowledgements

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