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Hybridization chain reaction engineered DNA nanopolylinker for amplified electrochemical sensing of biomarkers

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A DNA nanopolylinker was designed as a three dimensional nanoprobe with high loading of signal molecules for amplifying the biosensing signal. The nanoprobe was prepared by hybridization chain reaction engineering dsDNA polymerization on initiator DNA modified Au nanoparticle with two kinds of small molecule, for example, FITC-labeled DNA hairpins. The core–shell conjugate that was formed contained approximately 320 FITC molecules for further binding of signal molecules. With a sandwich-type immunoreaction and a biotin-streptavidin affinity reaction, the biotinylated core–shell nanoprobe was immobilized on the immunosensor surface, and the FITC molecules then bound enzyme labeled anti-FITC antibody to catalyze a silver deposition process, leading to a novel cascade signal amplification strategy. By combining the proposed strategy with stripping analysis of the deposited silver, an ultrasensitive immunoassay method for biomarker detection was developed. Under optimal conditions, this method showed a linear detection range over 5 orders of magnitude for carcinoembryonic antigen with a detection limit of 1.2 fg mL^{-1} (about 18 molecules in 5.0 μL sample). The preparation of DNA nanopolylinker was simple and economic, and it could be used as a universal and multifarious probe for different bioanalytical techniques and showed the promising potential of the signal amplification strategy in the future design of biosensing methodology.

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1 Introduction

A report probe that is normally used to signal biodetection events and measure the concentration of target molecules is one of the most import elements of biosensing systems. In order to achieve ultrasensitive and reliable detection, the report probe needs to be capable of generating reporting signals with a high signal-to-noise ratio. Advanced probes including fluorophores,[1–3] radioisotopes,[4] enzymes[5,6] and electroactive molecules[7,8] have been developed and widely used in different bioassays. However, these probes still face the challenge to detect biomarkers at ultralow levels due to their low signal intensities.

Recently, great effects have focused on the development of highly efficient probes with a strong reporting signal. The most popular strategy is to load large numbers of reporting molecules on nanocarriers, such as nanoparticles,[9,10] carbon nanotubes,[11,12] graphene,[13] and magnetic beads.[14,15] Gold nanoparticles (AuNPs) are the most used nanocarriers due to the advantages such as convenient preparation, good stability and biocompatibility and easy functionalization with proteins.[16] In particular, AuNPs can be modified with enzymes or enzyme labeled antibodies to prepare multienzyme nanoprobes and construct enhanced immunoassays for low-level proteins.[17–19] However, the amplification ability of these probes was limited due to the limited space for loading signal molecules. In order to overcome the low loading ability of AuNPs, Rusling *et al.*[14] used 1 μm diameter magnetic beads to synthesize a magnetic bioconjugate probe, on which ~7500 horseradish peroxidase molecules were loaded, for labeling a secondary antibody and achieved a detection limit of 0.5 pg mL^{-1}. Unfortunately, the large diameter of the magnetic beads led to a linear range of only 2 orders of magnitude. Thus, novel probes with high loading of signal molecules are urgently needed for achieving both high sensitivity and a wide detection range.

The biobarcode probe invented by Mirkin's group is the classic report probe and has been widely used for signal amplification.[20–24] It is usually prepared by functionalizing AuNPs with a large number of oligonucleotide strands (the barcodes), in which the barcodes are used as a means of amplification to quantitatively detect the target. Normally, each biobarcode probe can contain ~100 barcodes,[25] leading to much higher detection sensitivity over other biodetection schemes using conventional probes.[26,27] Our previous works prepared a biobarcode based probe using DNAzyme, which contained ~50 DNAzymes.[28,29] This probe efficiently amplified the detection signal and offered the detection limit of

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biomarkers down to pg mL^{-1} and even to fg mL^{-1} levels in chemiluminescent and electrochemiluminescent assays, respectively. However, as preparation of the solid-core probes is stuck in the single-layer surface modification, the loading amount of signal molecules on the report probe as well as the amplification efficiency is still greatly limited.

In this work, a DNA nanopolylinker was designed as a three dimensional nanoprobe with high loading of signal molecules for amplifying the biosensing signal. The nanoprobe was prepared by rationally engineering dsDNA polymerization on initiator DNA modified AuNP *via* a hybridization chain reaction (HCR)[30] with two kinds of small molecule, for example, fluorescein isothiocyanate (FITC) labeled DNA hairpins (FITC-H1 and FITC-H2) (Fig. 1). Generally, under mild conditions each copy of the initiator can trigger a HCR event, resulting in the linkage of many oligonucleotides, this has been used successfully in DNA and protein detection.[31–35] The DNA shell was rich in FITC and could serve as the polylinker for binding different signal molecules *via* the affinity reaction between FITC and anti-FITC antibody. After binding the biotinylated complementary oligos to the sticky ends of the HCR polymerization, the biotinylated nanopolylinker could be conveniently used as a universal probe for biosensing. By combining the designed probe with the enzymatic silver deposition amplification, a cascade signal amplification strategy was thus proposed for ultrasensitive electrochemical immunosensing (Fig. 2). Here the signal amplification included: (1) highly loaded FITC and following enzyme molecules on nano-sized three dimensional core–shell conjugate, (2) enzymatic reaction for silver deposition, and (3) stripping analysis of deposited silver, which led to a wide linear range for biomarker analysis with a detection limit down to about 18 molecules in 5.0 μL sample.

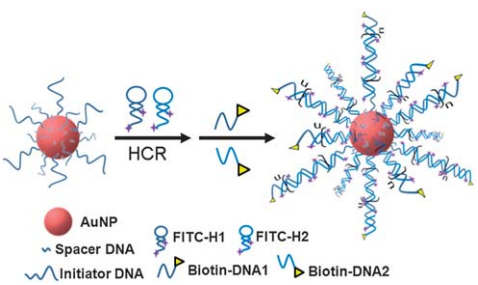

Fig. 1 Synthesis of the DNA nanopolylinker.

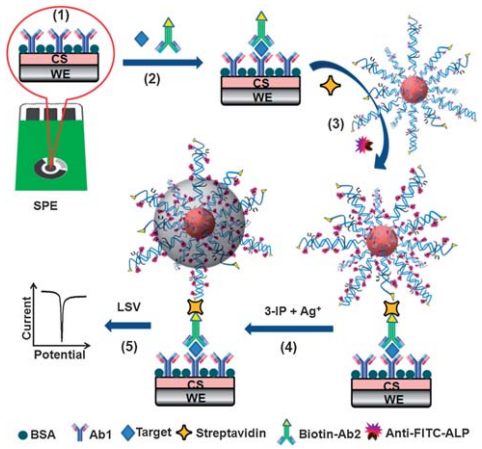

Fig. 2 Schematic diagram of the amplified electrochemical immunoassay using the DNA nanopolylinker probe: (1) structure of immunosensor, (2) sandwich-type immunoreaction of target protein and biotin-labeled antibody, (3) binding of the nanopolylinker probe to immunocomplex through streptavidin-biotin reaction and ALP-labeled anti-FITC to the probe, (4) enzymatic induced silver deposition, and (5) linear sweep voltammetry for stripping analysis of the deposited AgNPs.

2 Experimental

2.1 Materials and reagents

Capture antibody of carcinoembryonic antigen (CEA) (Ab1) was purchased from Shuangliu Zhenglong Biochem. Lab (Chengdu, China). Biotinylated anti-CEA (biotin-Ab2) and CEA standard solutions with concentrations from 0 to 75 ng mL^{-1} were from a commercial CEA ELISA kit, which was supplied by Fujirebio Diagnostics AB (Goteborg, Sweden). Chitosan (CS, ≥95% deacetylation), glutaraldehyde (GA, 25%), bovine serum albumin (BSA), anti-fluorescein-alkaline phosphatase antibody produced in rabbit (anti-FITC-ALP) and 3-indoxyl phosphate (3-IP) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-FITC-ALP was diluted 2000 times before use. ALP-labeled streptavidin was obtained from Beyotime Biotechnology (Haimen, China). Streptavidin was obtained from Promega (USA). Chloroauric acid ($HAuCl_4 \cdot 4H_2O$), $AgNO_3$, $Mg(NO_3)_2$, and trisodium citrate were obtained from Shanghai Reagent Co. (Shanghai, China). Ultrapure water obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore) was used in all assays. Clinical serum samples were from Jiangsu Institute of Cancer Prevention and Cure. All other reagents were of analytical grade and used as received.

Tris–HNO_3 buffer (TB) (10 mM, pH 7.4) containing 1 M NaCl was used for HCR. 1× TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was used for the storage of all DNA sequences. TB (100 mM, pH 7.4) was used as the reaction buffer for immunoassay. TB (100 mM, pH 7.4) containing 0.05% (w/v) Tween-20 and 5% (w/v) BSA were used as washing and blocking buffer, respectively. A solution mixture of 10 mM 3-IP and 1.5 mM $AgNO_3$ was prepared daily for silver deposition in TB (100 mM, pH 9.8) containing 20 mM $Mg(NO_3)_2$ and stored in an opaque tube at 4 °C.

The initiator DNA, spacer DNA and biotinylated oligos (biotin-DNA1 and biotin-DNA2) were purchased from Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China), and the H1 and H2 hairpins that were dual-labeled with FITC at each end (FITC-H1, FITC-H2) were purchased from Sunbiotech Co., Ltd. (Beijing, China). The sequences of all oligonucleotides were listed as follows:

Initiator DNA: 5′-AGTCTAGGATTCGGCGTGGGTTAA T_{15}-SH-3′

Spacer DNA: 5′-SH-T_{15}-3′

FITC-H1: 5′-FITC-TTAACCCACGCCGAATCCTAGACT *CAAA GT* AGTCTAGGATTCGGCGTG-FITC-3′

FITC-H2: 5′-FITC-AGTCTAGGATTCGGCGTG*GGTTAA* CA CGCCGAATCCTAGACT ACTTTG-FITC-3′

Biotin-DNA1: 5′-TTAACCCACGCCGAATCCTAGACTT$_5$-biotin-3′

Biotin-DNA2: 5′-biotin-T$_5$CACGCCGAATCCTAGACTACTT TG-3′

Prior to use, all the hairpins were heated to 90 °C for 90 s, and then allowed to cool to room temperature for 1 h.

2.2 Apparatus

The fluorescence spectrum was observed with a F900 fluorescence spectrometer (Edinburgh Instruments Ltd., U.K.). Dynamic light scattering (DLS) measurement was performed using a BI-200SM light scattering apparatus (Brookhaven, U.S.A.). All electrochemical measurements were performed using a CHI 660B electrochemical workstation (CHI, Shanghai, China). The reference immunoassay was performed with a Roche Elecsys 2010 immunoassay analyzer (Roche Diagnostics GmbH).

2.3 Preparation of biotinylated DNA nanopolylinker

AuNPs with 13 nm diameter were prepared according to the previous protocol.[36] 15 μL of the mixture (60 μM) of initiator and spacer DNA at a ratio of 1 : 5 was added into 200 μL of 5.0 nM AuNP solution and incubated for 0.5 h. Then 24 μL of 100 mM TB (pH 7.4) containing 0.1% Tween-20 was added to the mixture and incubated at room temperature for 20 min. Small aliquots of 2.0 M NaCl in 10 mM TB containing 0.01% Tween-20 were added stepwise to raise the NaCl concentration to 1.0 M, during which a 10 s sonication and a 20 min incubation were required for each addition step of NaCl. The mixture was incubated overnight at room temperature.[25] Subsequently, a centrifugation process was performed to remove the excess oligonucleotides and obtain the initiator DNA modified AuNPs, which were resuspended in 200 μL 10 mM TB containing 1.0 M NaCl and 0.01% Tween-20.

A 600 μL mixture of 0.2 μM FITC-H1 and FITC-H2 was mixed with 200 μL of initiator DNA modified AuNP solution and gently shaken for 4 h to form a three-dimensional HCR product. The excess hairpins were removed by centrifugation. The obtained HCR product was redispersed in 200 μL 10 mM TB containing 1.0 M NaCl and 0.01% Tween-20. Then, 10 μL solutions of 10 μM biotin-DNA1 and biotin-DNA2 were simultaneously added to 200 μL of the HCR product and reacted for 40 min to form a biotinylated DNA nanopolylinker probe (Fig. 1). After removing the excess biotinylated oligos by centrifugation, the obtained nanopolylinker probe was redispersed in 10 mM TB containing 1.0 M NaCl, and stored at 4 °C prior to use.

In order to determine the amount of FITC on each DNA nanopolylinker, the FITC-H1 and FITC-H2 were firstly dissociated from the nanopolylinker by mixing equal volumes of HCR product with 0.1 M NaOH along with 4 h incubation at room temperature. After the AuNP precipitate was removed by centrifugation, the supernatant solution was neutralized with 0.1 M HCl. The concentration of AuNPs was determined using UV-vis spectroscopy,[37] and the concentration of FITC was obtained by fluorescent spectroscopy.[38]

2.4 Analytical protocol

The proposed electrochemical immunoassay was illustrated in (Fig. 2). The immunosensing platform was constructed on a disposable screen-printed carbon electrode (SPE), on which Ab1 was covalently assembled on the CS modified working electrode through GA cross-linking.[39] A sandwich-type immunoassay was performed by sequentially incubating 5 μL of CEA standard solution (or serum sample) and 10 μL of biotin-Ab2 on the immunosensor for 30 min. Then, 10 μL of 10 μg mL^{-1} streptavidin, biotinylated DNA nanopolylinker probe, and anti-FITC-ALP were dropped sequentially on the immunosensor to react for 20 min, 20 min and 40 min, respectively. Next, 10 μL of silver deposition solution was delivered to the immunosensor surface for 30 min, which was protected from light. Finally, a linear sweep voltammetric (LSV) detection from −0.15 to +0.30 V at 50 mV s^{-1} was performed in a 1.0 M KCl solution to record the stripping current for detection of CEA.

3 Results and discussion

3.1 Condition optimization for preparation of DNA nanopolylinker

The goal of this work is to develop a probe with high loading of enzyme for constructing an amplified electrochemical sensing platform for biomarkers. The preparation of the nanoploylinker is based on the growth of dsDNA polymer through *in situ* HCR on the surface of AuNPs. By using the FITC labeled hairpins, numerous FITC molecules are loaded on the DNA assembled AuNPs to serve as enzyme linkers though the recognition reaction between FITC and ALP-labeled anti-FITC. Thus, combining the novel DNA nanopolylinker with the ALP based silver deposition amplification, a sensitive immunoassay method could be constructed (Fig. 2).

The preparation of three-dimensional DNA nanopolylinker employed initiator DNA self-assembled AuNPs to trigger the HCR. Thus, the control of the orientation and coverage of initiator DNA on AuNPs was essential. This work used a short poly-T DNA as a spacer to "stand up" the initiator DNA and tune its coverage for achieving high hybridization performance.[25] The ratio of initiator DNA to spacer DNA was first optimized by examining the amount of FITC-H1 hybridized with the initiator DNA modified AuNPs. After dissociating FITC-H1 from the FITC-H1 hybridized AuNPs with 0.1 M NaOH and separating the FITC-H1 from the mixture, the fluorescence intensity of the supernatant solution changed with the varying ratio of initiator DNA to spacer DNA (Fig. 3A), and showed a maximum value at a ratio of 1 : 5. Obviously, with the increasing amount of spacer DNA, more spacer DNA was assembled on the AuNP to efficiently improve the orientation and spacing of initiator DNA, resulting in an increase of FITC-H1 hybridized on the AuNPs. Too much spacer DNA decreased the coverage of the initiator DNA, and thus decreased the amount of hybridized FITC-H1.

The concentration of hairpins for HCR was also optimized by detecting the amount of FITC bound on the HCR product. In this work, FITC-H1 and FITC-H2 were used at equal concentration. As shown in Fig. 3B, the fluorescence intensity of FITC

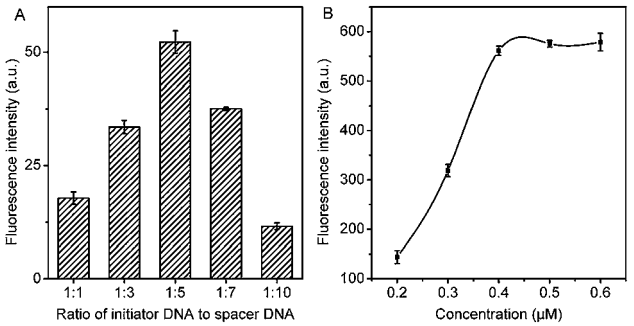

Fig. 3 Effect of (A) ratio of initiator DNA to spacer DNA and (B) concentration of hairpins on the amount of FITC in formed DNA nanopolylinker ($n = 3$ for error bars).

in the supernatant solution increased with increasing concentrations of hairpins and reached a plateau at 0.4 μM, indicating HCR reached an equilibrium state. Thus, 0.4 μM of FITC-H1 and FITC-H2 were used for the preparation of DNA nanopolylinker probes.

3.2 Characterization of DNA nanopolylinker

The HCR-based DNA assembly on AuNPs was characterized by fluorescence spectra (Fig. 4A). In the absence of FITC-H2, the formed FITC-H1 hybridized AuNPs (curve b) showed much lower fluorescence intensity of FITC in the supernatant solution than that formed in the presence of FITC-H2 (curve a), indicating that more FITC molecules were brought to the surface of AuNPs by the alternate hybridization between FITC-H1 and FITC-H2.

The coverage of active initiator DNA on a single AuNP was determined to be 20 by dissociating the FITC-H1 from FITC-H1 hybridized AuNPs and then measuring the concentration of FITC-H1 in the supernatant solution and the amount of AuNPs.[38] Similarly, the number of FITC on each DNA nanopolylinker was estimated to be about 320, *i.e.* 80 pairs of FITC-H1 and FITC-H2 were assembled on each AuNP. Thus, the average cycle number of HCR (every cycle includes one alternate hybridization of H1 and H2 with two labeled ends) triggered by one active initiator DNA on the surface of AuNP was 4. This result indicated the nanopolylinker probe could bind ~320 enzyme molecules by the affinity reaction between FITC and enzyme-labeled anti-FITC antibody. Thus, the surface enzyme coverage of the proposed DNA nanopolylinker was estimated to be 0.6 enzyme per nm^2, which was much higher than those of 0.002, 0.02 and 0.1 enzyme per nm^2 for HRP-antibody/magnetic bead,[14] HRP-antibody/AuNP[18] and DNAzyme/AuNP labels,[29] respectively, showing the possibility to achieve both high sensitivity and a wide detection range with the immunoassay.

Dynamic light scattering measurements showed that the AuNPs had an average hydrodynamic diameter of 13(±2.4) nm (Fig. 4B-a), and the average hydrodynamic diameter of FITC-H1 hybridized AuNPs was 30(±3.7) nm (Fig. 4B-b). After HCR and reaction with biotin-DNA1 and biotin-DNA2, the average hydrodynamic diameter increased to 72(±5.3) nm (Fig. 4B-c), suggesting the successful formation of a DNA polymerization shell on the AuNP surface. These results also confirmed that the prepared DNA nanopolylinker possessed good dispersity in aqueous media and no aggregates or precipitates emerged, which was essential for the biological application.

The nanopolylinker probe could be stored at 4 °C when out of use. After a storage period of 15 days, the response for electrochemical immunoassay of CEA remained 94% of the initial value, indicating that the probe had acceptable stability.

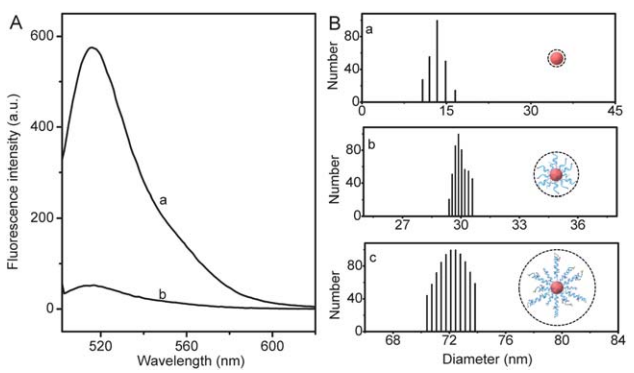

Fig. 4 (A) Fluorescence spectra of FITC in the supernatant solution obtained from (a) DNA nanopolylinker and (b) FITC-H1 hybridized AuNPs, and (B) hydrodynamic diameter of (a) AuNP in water, (b) FITC-H1 hybridized AuNPs, and (c) DNA nanopolylinker probe in 10 mM TB buffer (pH 7.4) containing 0.5 M NaCl.

3.3 Optimization of detection conditions

After the DNA nanopolylinker was biotinylated with biotin-DNA1 and biotin-DNA2, the obtained probe could be easily bound to the target-related immunocomplex or hybridization product *via* biotin-streptavidin affinity reaction for protein or DNA detection. Using CEA as a model biomarker, the DNA nanopolylinker probe could be bound to the surface of CEA immunosensor *via* sandwich-type immunoreaction and the following biotin-streptavidin recognition, which was then incubated in ALP-labeled anti-FITC solution to bind enzyme molecules onto the immunosensor. Thus, the incubation time of ALP-labeled anti-FITC was first optimized. As shown in Fig. 5A, the stripping peak current of enzymatically deposited silver increased sharply with the increasing incubation time and tended to level off after 40 min, indicating saturation of the capture of anti-FITC-ALP on the nanopolylinker probe surface. Therefore, an incubation time of 40 min was selected for the conjugation of ALP-labeled anti-FITC.

The sensitivity of the proposed immunoassay method was dependent on the ALP based silver deposition amplification (Fig. 2). Thus factors that affect sensitivity include concentration of 3-IP and silver cation and the deposition time. At a deposition time of 30 min, the stripping current reached maximum values at 3-IP and $AgNO_3$ concentrations of 10 mM (Fig. 5B) and 1.5 mM (Fig. 5C), respectively. This phenomena indicated that 10 mM 3-IP and 1.5 mM $AgNO_3$ were sufficient for the proposed ALP-induced silver deposition procedure, at which

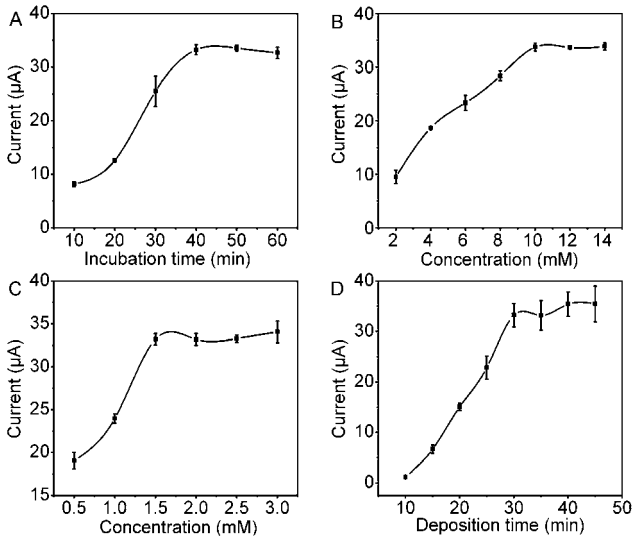

Fig. 5 Effects of (A) incubation time for ALP-labeled anti-FITC, concentrations of (B) 3-IP and (C) $AgNO_3$, and (D) silver deposition time on stripping current of AgNPs at 1 ng mL^{-1} CEA in 1.0 M KCl ($n = 3$ for error bars).

the stripping current increased rapidly with the deposition time upto 30 min (Fig. 5D), indicating 30 min was the optimal deposition time for the immunoassay.

3.4 Amplification properties of the DNA nanopolylinker

In order to evaluate the amplification ability of the proposed DNA nanopolylinker probe, the sandwich-type immunoassays of 0.1 ng mL^{-1} CEA using the proposed DNA nanopolylinker, FITC-H1 hybridized AuNPs and ALP-labeled streptavidin probes were performed. As shown in Fig. 6, the nanopolylinker probe produced 2.2 and 4.0 times higher current responses compared to FITC-H1 hybridized AuNPs and ALP-labeled streptavidin, respectively, along with similar noise. Moreover, with decreasing CEA concentration, the difference between the signal-to-noise ratio for the nanopolylinker probe and FITC-H1 hybridized AuNPs became greater, indicating the advantage of the nanopolylinker probe for immunoassay of low-abundance biomarkers.

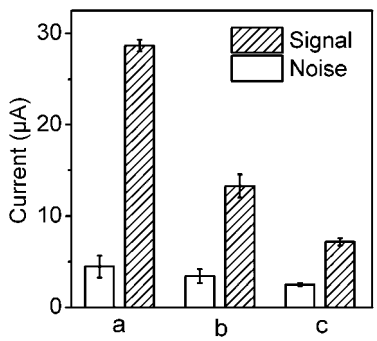

Fig. 6 LSV responses at 0.1 ng mL^{-1} CEA using (a) DNA nanopolylinker probe, (b) FITC-H1 hybridized AuNPs and (c) ALP-labeled streptavidin ($n = 3$ for error bars).

3.5 Analytical performance

Under optimum conditions, the stripping peak current of the deposited Ag increased with increasing concentration of CEA in the incubation solution for sandwich-type immunoreaction (Fig. 7A). The calibration plot showed a good linear relationship between the current response and the logarithm value of the CEA concentration in the range of 0.01 pg mL^{-1} to 1 ng mL^{-1} with a correlation coefficient of 0.9993 (Fig. 7B). The detection limit corresponding to a signal-to-noise ratio of 3 was 1.2 fg mL^{-1}, which was about 18 molecules in 5.0 μL sample and much lower than those reported previously.[14,40–42] The sensitivity, determined from the slope of the calibration curve was 4.64 μA mL ng^{-1}, which was much larger than that reported previously in immunoassays using ALP-antibody functionalized AuNP probes.[39] The ultralow detection limit, high sensitivity and wide linear range over 5 orders of magnitude indicated promising application of the proposed DNA nanopolylinker and amplified biosensing platform.

The non-specific binding characteristic of the proposed immunoassay was evaluated by comparing the current response toward solutions containing either CEA or other antigen only, for example α-fetoprotein (AFP), or a mixture of CEA and AFP (Fig. 8). As expected, the immunosensor showed obvious responses to only the solutions containing target CEA (column c and d), while negligible response were observed in the AFP solution (column b), indicating little non-specific binding occurred between the immunosensor and non-specific antigen.

3.6 Detection of CEA in serum samples

To evaluate the analytical reliability and application potential of the proposed immunoassay, the assay results of CEA in clinical serum samples obtained by the proposed immunoassay were compared with the reference values from the commercial electrochemiluminescent testing. Due to the high detection sensitivity of the proposed method, serum samples were diluted 100 or 500 times prior to assay. The results were shown in Table 1, an acceptable agreement with relative errors less than 9.7% indicated good accuracy of the proposed method for the detection of clinical samples.

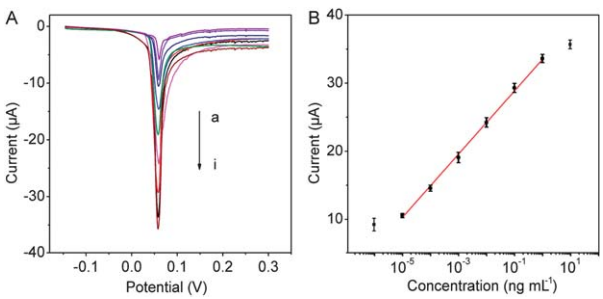

Fig. 7 (A) LSV responses and (B) calibration curve of the proposed method for CEA detection. Curves a–i are for 0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 0.1, 1, 10 ng mL^{-1} CEA ($n = 3$ for error bars).

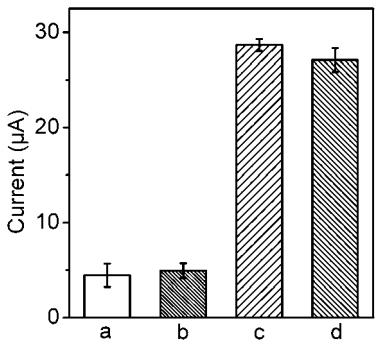

Fig. 8 LSV responses of immunosensor for (a) blank control, (b) 0.1 ng mL^{-1} AFP, (c) 0.1 ng mL^{-1} CEA, and (d) the mixture of 0.1 ng mL^{-1} CEA and 0.1 ng mL^{-1} AFP ($n = 3$ for error bars).

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

Sample	Proposed method	Reference method	Relative error (%)
1	0.8875[a]	430.2	3.1
2	0.3931[a]	40.2	−2.2
3	0.1973[a]	18.6	6.1
4	0.6240[a]	336.6	−7.3
5	0.2467[a]	136.6	−9.7

[a] The serum samples were diluted at 500 and 100 times, respectively, prior to assay.

4 Conclusion

This work developed a DNA nanopolylinker probe, a three dimensional core–shell conjugate, for the amplified electrochemical sensing of biomarkers. The nanopolylinker was prepared by rationally engineering dsDNA polymerization on AuNP *via* HCR. The nanopolylinker had a diameter of 72 nm and contained approximately 320 binding sites of signal molecules to significantly enhance the reporting signals. By combining the DNA nanoploylinker probe with the enzymatic silver deposition amplification, an ultrasensitive electrochemical immunosensing platform was constructed, which showed a wide linear detection range and a detection limit of 1.2 fg mL^{-1} for CEA. The DNA nanopolylinker could be simply and economically prepared, and the cascade signal amplification strategy could be conveniently used for the design of other biosensing methods by employing different binding reactions.

Acknowledgements

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