Analytica Chimica Acta 1186 (2021) 339083

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

A DNA dendrimer amplified electrochemical immunosensing method for highly sensitive detection of prostate specific antigen

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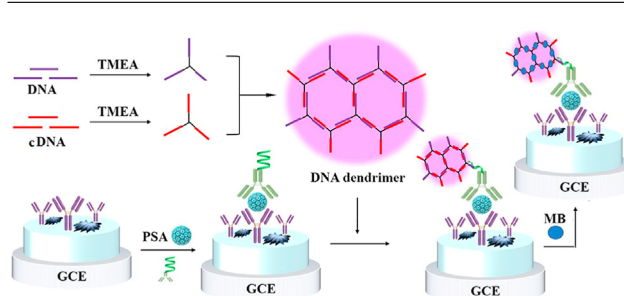
HIGHLIGHTS

- A DNA dendrimer is designed for loading of a large amount of electro-active molecule to amplify the immunosensing signal.
- The DNA dendrimer can be conveniently assembled with a couple of complementary Y-shaped DNAs.
- The proposed immunosensing method shows a wide concentration range and a limit of detection down to sub-pg·mL^{-1}.
- The good selectivity and accuracy for serum sample analysis demonstrate the potential application of the DNA dendrimer.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 31 August 2021
Received in revised form
17 September 2021
Accepted 18 September 2021
Available online 25 September 2021

Keywords:
Electrochemical sensors
Immunoassay
DNA dendrimer
Biosensor
Signal amplification
Prostate specific antigen

ABSTRACT

This work designed a DNA dendrimer for the loading of signal molecule and the construction of amplified electrochemical immunosensing method. The DNA dendrimer was self-assembled by the hybridization of one couple of complementary oligonucleotides (DNA and cDNA) that were covalently conjugated to three arms of a Y-shaped cross-linker, tris(2-maleimidoethyl)amine (TMEA) respectively. The immunosensor was prepared by coating chitosan on glassy carbon electrode to covalently immobilize the capture antibody with glutaraldehyde as a linker. After the target protein was captured on the immunosensor, cDNA-labeled secondary antibody was bound on the surface via a sandwiched immunoreaction to introduce the DNA dendrimer onto immunosensor for loading abundant methylene blue as signal molecule, which amplified greatly the amperometric signal for immunoassay. Using prostate specific antigen (PSA) as a model analyte, this proposed method showed a wide linear range from 1 pg mL^{-1} to 10 ng mL^{-1} along with a limit of detection down to 0.26 pg mL^{-1}. The designed strategy avoided complex synthesis of signal tags, and possessed excellent performance for analysis of practical samples, thus providing a new avenue for the development of signal amplification strategy and immunoassay methods.
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1. Introduction

High mortality rate of malignant cancer is threatening human health. Early diagnosis and treatment are a key to improve the

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https://doi.org/10.1016/j.aca.2021.339083
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survival rate of cancer patients [1]. Accurate and sensitive detection of protein biomarkers plays a significant role in early cancer screening and diagnosis [2]. Immunoassay based on antigen-antibody interaction has been viewed as an important analytical method for quantitative determination of protein biomarkers due to the highly specific molecular recognition for immunoreaction [3]. Various measurement technologies such as fluorescence [4], chemiluminescence [5], surface-plasmon resonance [6] and quartz crystal microbalance [7] have been applied to immunoassay [8]. Comparing with these technologies, electrochemical immunoassay has a lot of advantages, such as easy operation, low cast, high sensitivity, simple instrument and fast detection [9]. Moreover, it is very easy to combine with signal amplification strategies based on enzyme catalysis, nanomaterials, and DNA hybridization for highly sensitive detection of low-abundant biomarkers in early cancer diagnosis [10–13]. Usually nanomaterials-based signal amplification in electrochemical immunoassay includes: 1) using nanomaterials to accelerate electron transfer, 2) using nanomaterials as enzyme mimic, and 3) using nanomaterials as signal tags or the carriers of enzyme or signal molecules [14]. Unfortunately, the toxicity of some nanomaterials limits the practical application of related immunoassay methods. In this work, we designed a nontoxic DNA dendrimer for the loading of signal molecule to develop a highly sensitive electrochemical immunosensing method for the analysis of prostate specific antigen (PSA).

Dendrimers, discovered in the late 1970's, are highly branched, monodisperse, nanosized, globular, and void-containing polymers [15]. These polymers have been used as carriers of signal molecules for design of signal amplification strategies [16]. Owing to the programmable sequences and controllable length, DNA has been used for fabrication of dendrimer-like structures using enzymatic ligation [17] or step-by-step assembly of three-armed DNA monomers [18]. This kind of structures has been considered as an ideal carrier of signal molecules to improve the sensitivity of fluorescence [19,20], surface-enhanced Raman scattering [21], chemiluminescence [22], quartz crystal microbalance [23], amperometric [24] and electrochemiluminescence [25] methods. By assembling a large number of quantum dots (QDs) onto the dendrimer-like DNA structure, the amplified amperometric detection showed high sensitivity [24]. However, the assembly of QDs modified with a couple of complementary DNAs severely influenced the detection repeatability. Moreover, the on-site assembly led to tedious detection process. In this work, the DNA dendrimer was simply prepared by the hybridization of one couple of complementary oligonucleotides (DNA and cDNA) that were covalently conjugated to three arms of a Y-shaped tris(2-maleimidoethyl) amine (TMEA) respectively [26]. After the dendrimer was bound to immunosensor by the cDNA-labeled secondary antibody, it was used as the carrier to load abundant electroactive molecules, methylene blue, for signal amplification (Fig. 1), which greatly simplified the dendrimer-based signal amplification strategy.

In this work, PSA was selected as the sensing target since it is a biomarker of prostate cancer, and has come into widespread use to clinical testing, both for initial diagnosis and for monitoring of response to treatment [27]. The PSA level in the blood of health man is less than 4 ng mL^{-1} [28]. Different methods based on its recognition to antibody [29–34], peptide [35,36] or aptamer [37] have been developed for colorimetric [29–32,35], electrochemical [34,36] and photoelectrochemical [33,37] detection of PSA. Herein, the methylene blue loaded DNA dendrimer showed amplified electrochemical response for sandwich-type immunoassay of PSA. The excellent performance with a wide concentration range, a detection limit at sub-pg$\cdot$$mL^{-1}$ level, good selectivity and acceptable accuracy indicated that the designed immunoassay method along with the signal amplification strategy possessed the potential application in clinical diagnosis.

2. Experimental

2.1. Materials and reagents

The complementary oligonucleotides HS-C_6-AATCCGTCGAGCAGAGTT (DNA) and HS-C_6-AACTCTGCTCGACGGATT (cDNA) were supplied by Sangon Biological Engineering Technology & Co., Ltd. (Shanghai, China). Tris (2-maleimidoethyl)amine (TMEA) was purchased from Heowns Biochem Technologies LLC (Tianjin, China). Prostate specific antigen (PSA), monoclonal PSA captured antibody (Ab_1) and the secondary antibody (Ab_2) were supplied by Beijing Key-Bio Biotech Co., Ltd. (Beijing, China). Chitosan (CS) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Glutaraldehyde (GA, 25% aqueous solution) was purchased from Alfa Aesar China Ltd. Maleimidobenzoic acid *N*-hydroxy succinimide ester (MBS), methylene blue (MB), dimethyl sulfoxide (DMSO), and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich (St Louis, MO, USA). The serum samples were supplied by Jiangsu Cancer Hospital.

2.2. Apparatus

The cyclic voltammetry (CV) and square wave voltammetry (SWV) were performed on CHI630D electrochemical workstation (China). The electrochemical impedance spectra (EIS) were obtained with Donghua DH7000 electrochemical analyzer (China). Polyacrylamide gel electrophoresis (PAGE) analysis was carried out with an electrophoresis analyzer (Bio-Rad, USA) and a Bio-Rad ChemiDoc XRS (Bio-Rad, USA). Scanning electron microscope (SEM) (JEOL Model JSM-6490, Japan) was used to characterize the DNA dendrimer.

2.3. Preparation of DNA dendrimer

The DNA dendrimer was prepared via self-assembly of two complete complementary DNAs modified with TMEA according to the previous report [26] with slight modification. The thiol groups in DNA and cDNA were firstly activated with TECP at the molar ratio of 1:200 at room temperature for 2 h. After purified by ultrafiltration, the activated DNAs were mixed with TMEA at the molar ratio of 1:3 at room temperature overnight, during which the maleimidoethyl groups in three arms of TMEA reacted with thiol groups at the 5′-end of DNA respectively to obtain the Y-shaped trimers (Y-DNA and Y-cDNA), respectively, which were purified with 15% PAGE and gel extraction kit. The DNA dendrimer was then assembled with the Y-DNA and Y-cDNA at the ratio of 3:2 in 10 mM phosphate buffered saline (PBS, pH 7.4) at 37 °C for 1 h, which yielded the sticky ends DNA on the outermost layer of dendrimer for binding with cDNA-Ab_2 on immunosensor. Finally, the obtained DNA dendrimer was purified through ultrafiltration, dispersed in PBS (pH 7.4) and stored at 4 °C for further use.

2.4. Preparation of cDNA-Ab_2

The DNA was conjugated to the secondary antibody with MBS as the cross-linker according to the previous report [38] with slight modification. In MBS the *N*-hydroxysuccinimide ester group reacted with the amino group of Ab_2, and the maleimide group reacted with the thiol group at 5′-end of cDNA. 0.4 mg mL^{-1} Ab_2 was mixed with 0.8 mg mL^{-1} of MBS in PBS (pH 7.4) to incubate at room temperature for 2 h, and the superfluous MBS was removed by ultrafiltration. Meanwhile, 30 μL 100 μM cDNA was activated with TCEP at the molar ratio of 1:200 at room temperature for 2 h, and purified by ultrafiltration. The activated Ab_2 and cDNA were then

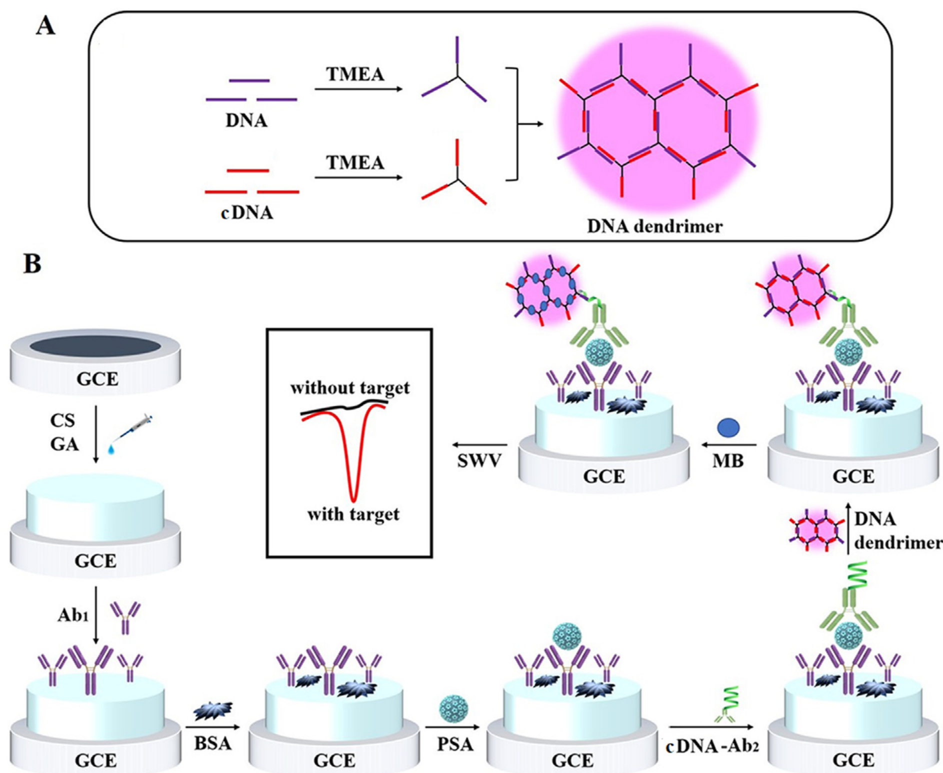

Fig. 1. Schematic illustration of (A) preparation of DNA dendrimer and (B) fabrication of immunosensor and electrochemical immunoassay process for PSA.

mixed to react for 2 h at room temperature. The obtained cDNA-Ab_2 conjugate was purified by ultrafiltration, re-dispersed in PBS and stored at 4 °C.

2.5. Preparation of immunosensor

The immunosensor was constructed by covalently immobilizing the capture antibody on a glassy carbon electrode through CS coating and GA cross-linking. Primarily, the bare GCE was polished with alumina slurry and sonicated for a completely clean surface. Then, 5 μL of 0.25 mg mL^{-1} CS was dropped on the electrode and dried at room temperature, followed by dropping 5 μL of 2.5% wt GA for 2 h and rinsing with deionized water. Next, 5 μL of 0.5 mg mL^{-1} Ab_1 was incubated on the electrode at room temperature for 1 h and then at 4 °C overnight in a 100% moisture-saturated environment. After washing with PBS to remove the excess Ab_1, 5 μL of 0.5% wt BSA was dropped on the Ab_1 modified GCE to incubate at room temperature for 1 h for blocking possible active sites against nonspecific absorption. The obtained immunosensor was rinsed with PBS for further use.

2.6. SWV detection of PSA

Sandwich-type immunoassay method was used for SWV detection of PSA. Firstly, 5 μL of standard solutions or serum samples were incubated on the immunosensors for 40 min at room temperature, respectively. After rinsing with PBS, 5 μL cDNA-Ab_2 was added on the immunosensor to incubate for 40 min and washed with PBS. Next, 5 μL of DNA dendrimer was dropped on the electrode to incubate for 1 h at 37 °C and rinsed again with PBS. Finally, 5 μL of 0.4 mM MB was dropped on the immunosensor for 15 min and washed with PBS again. The obtained biosensor was measured via SWV in 10 mM pH 7.4 PBS containing 0.1 M KCl from −0.7 V to 0.25 V (vs. Ag/AgCl) at an potential increasement of 0.004 V, amplitude of 0.025 V and frequency of 15 Hz.

3. Results and discussion

3.1. Characterization of the DNA dendrimer

DNA, cDNA, and their products reacting with TMEA were analyzed using 15% PAGE (Fig. 2A). The single-stranded DNAs (lane 1 and lane 4) showed the lowest molecular weight and ran fast on the gel, while their product reacting with TMEA contained trimeric, dimeric and monomeric DNAs, which displayed 3 electrophoretic bands due to the different velocities (lane 2 and lane 5). The Y-shaped DNAs (lane 3 and lane 6) contained trimeric DNAs, and showed only one electrophoretic band at trimer. These results demonstrated the successful synthesis and good purity of the Y-shaped DNAs. After two kinds of the Y-shaped DNAs hybridized with each other to produce the three-dimensional meshy DNA dendrimers, SEM was employed to observe the size of the assembled DNA dendrimer, which showed nearly spherical morphology with an average diameter of 250 nm (Fig. 2B). Sample for SEM test was prepared by coating the DNA dendrimer on ITO glass, and then spraying a gold layer. This image suggested the successful formation of the DNA dendrimer.

3.2. Characterization of cDNA-Ab_2

The preparation of cDNA-Ab_2 was also characterized using 15% PAGE. cDNA showed two bright bands on the gel due to the presence of some dimeric cDNAs that were formed via the sulfhydryl group of the cDNA. Ab_2 did not show any band due to its low sensitivity to UltraPowerTM dye for PAGE analysis. However, after the activated Ab_2 and cDNA were mixed to react for 2 h, the purified

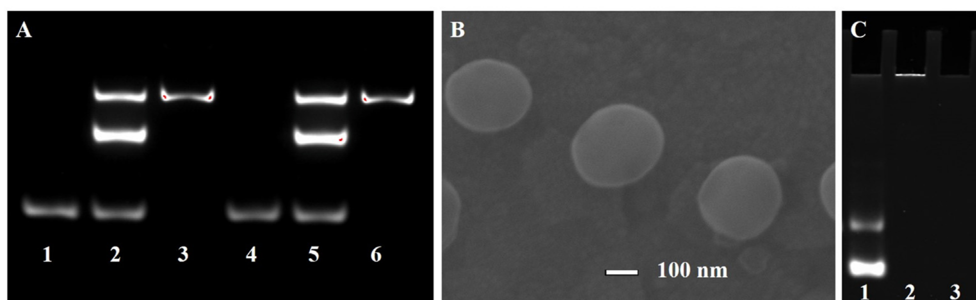

Fig. 2. (A) PAGE analysis of different DNA structures: lane 1, DNA, lane 2, products of DNA reacting with TMEA (contains trimeric, dimeric and monomeric DNAs), lane 3, Y-DNA (purified trimeric DNA), lane 4, cDNA, lane 5, product of cDNA reacting with TMEA (contains trimeric, dimeric and monomeric DNAs), lane 6, Y-cDNA (purified trimeric cDNA). (B) SEM image of the DNA dendrimer. (C) PAGE analysis of cDNA-Ab_2 structure: lane 1, cDNA, lane 2, cDNA-Ab_2; lane 3, Ab_2.

product showed a narrow band of DNA at large molecular weight (Fig. 2C, lane 2), which could be attributed to the presence of pure cDNA-Ab_2 conjugate.

3.3. Characterization of immunosensor

Both CV and EIS measurements in 10 mM pH 7.4 PBS containing 0.1 M KCl and 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1) were employed to confirm the process of the immunosensor fabrication. Compared with the CV peaks of $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ at bare GCE, the peak currents at electrode modified with CS slightly increased due to the presence of more positive charge on the electrode surface, which was in favor of the redox reaction of $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ at GCE, and thus leading to slight decrease of the separation of peak potentials. However, after the GEC/CS was further modified with GA, Ab_1 and BSA, the peak currents successively decreased and the separation of peak potentials successively increased (Fig. 3A), indicating the worse reversibility of the electrode reaction upon the binding of GA, Ab_1 and then BSA to electrode surface for obtaining the immunosensor. These results were also observed from the impedance spectra (Fig. 3B). The coating of CS on electrode surface decreased the electron transfer resistance, but the resistance consecutively increased with the following modification, demonstrated the loading of GA, Ab_1 and BSA on GEC/CS.

3.4. Optimization of incubation time

The incubation time is an important parameter affecting the analytical performance of immunoassay. To maximize the detection sensitivity, the incubation time for sandwich-type immunoreactions was optimized at room temperature. The SWV response to PSA increased with the increasing incubation time used in sandwich-type immunoassay and then tended to a constant value after 40 min (Fig. 4), which showed the saturated binding between antigen and antibody on immunosensor surface. Therefore, the optimum incubation time of 40 min was selected for the immunoassay.

3.5. Quantitative detection of PSA

To investigate the potential performance and the sensitivity of the proposed biosensor, we detected various concentrations of PSA

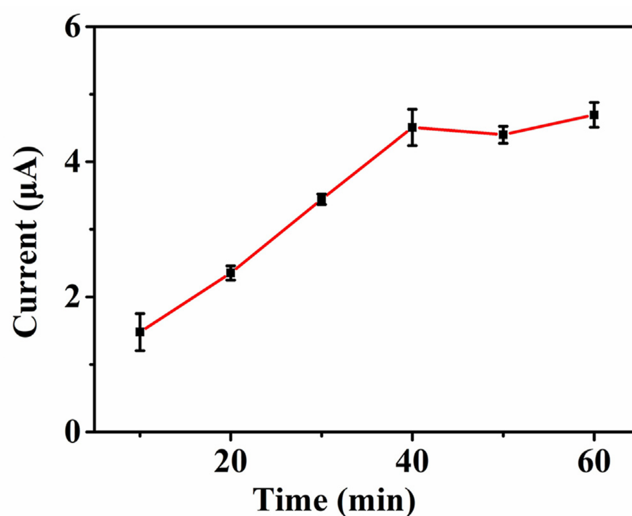

Fig. 4. Influence of incubation time on immunosensing response to 0.05 ng mL^{-1} PSA.

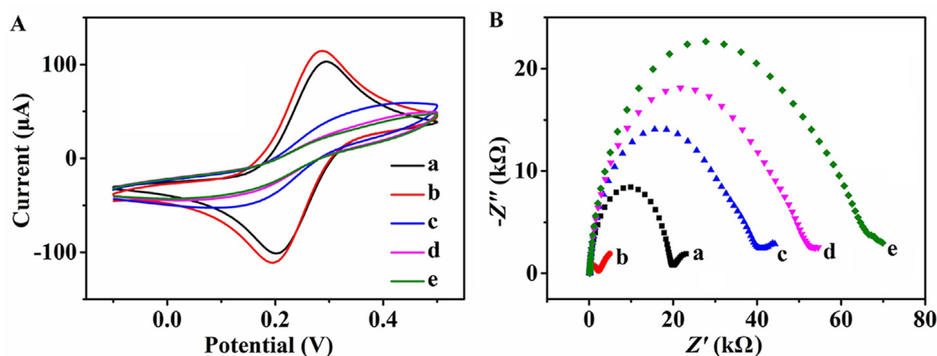

Fig. 3. (A) CV and (B) EIS characterization of immunosensor preparation: (a) GCE, (b) GCE/CS, (c) GCE/CS/GA, (d) GCE/CS/GA/Ab_1 and (e) GCE/CS/GA/Ab_1/BSA in 10 mM pH 7.4 PBS containing 0.1 M KCl and 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1). Frequency range: 100 kHz to 0.01 Hz; amplitude: 10 mV.

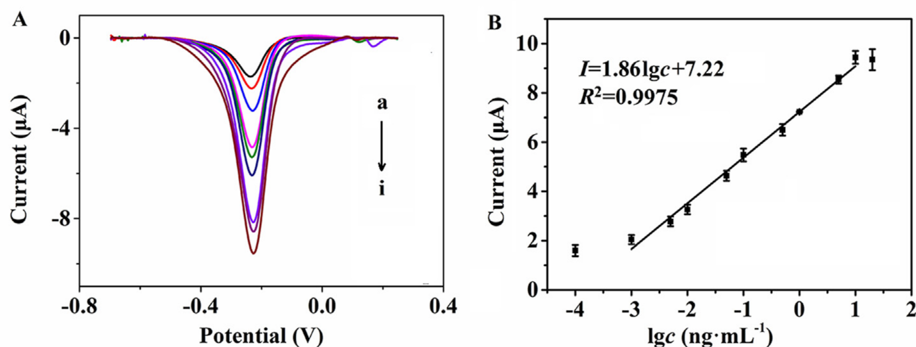

Fig. 5. (A) SWV curves of immunosensors for 0.001, (b) 0.005, (c) 0.01, (d) 0.05, (e) 0.1, (f) 0.5, (g) 1.0, (h) 5.0 and (i) 10 ng mL^{-1} PSA. (B) Calibration plot of the proposed immunosensor for PSA. Detection buffer: 10 mM pH 7.4 PBS containing 0.1 M KCl.

Table 1
Comparison of the performance and the signal amplification strategies of present method with the methods reported previously.

Method	Signal amplification strategy	Linear range (ng·mL^{-1})	LOD (ng·mL^{-1})	References
Electrochromic	Bi_2S_3 nanorods	1×10^{-1}-5×10^{1}	3×10^{-2}	[33]
PEC	Glucose-loading SiO_2NP	1×10^{-3}–1×10^{2}	3×10^{-4}	[37]
CV	Crumpled GR-Au ball	0–10	5.9×10^{-1}	[34]
Colorimetry	Enzymatic reaction	1×10^{-3}–1×10^{6}	4.6×10^{-4}	[29]
Colorimetry	Enzymatic reaction	0–10	4	[30]
Colorimetry	Enzymatic reaction	3×10^{-1}-3	9.3×10^{-3}	[31]
Colorimetry	AuNP-assisted copper deposition	0–1×10^{2}	3.4×10^{-3}	[32]
Colorimetry	Catalyzed oxidation	5×10^{-2}-8×10^{-1}	2×10^{-2}	[35]
EC	Enzyme catalysis	8×10^{-2}-7	3×10^{-2}	[36]
SWV	DNA dendrimer	1×10^{-3}–1×10^{1}	2.6×10^{-4}	This work

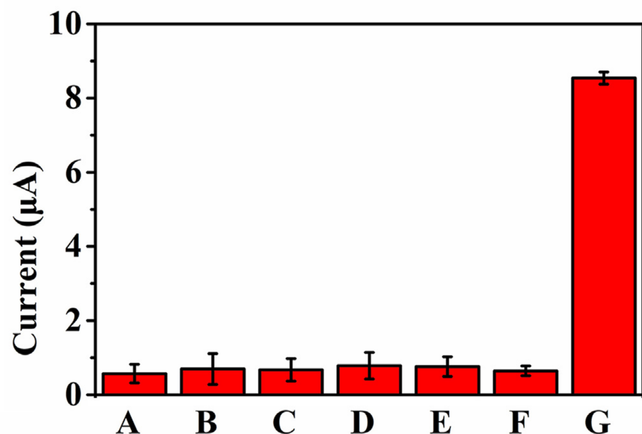

Fig. 6. The specificity evaluation of the immunosensor to (A) blank control, (B) AFP, (C) CEA, (D) BSA, (E) NT-proBNP, (F) SARS-CoV-2, and (G) PSA at 5 ng mL^{-1}.

using the prepared immunosensors. With a sandwich-type immunoassay format, the quantitatively captured MB on the immunosensors could be easily detected via SWV. Under the optimum condition, the peak current of the immunosensor for target PSA gradually increased with the increasing concentration of analytes (Fig. 5A). The calibration plot showed a good linear relationship between the peak current and the logarithm value of the analyte concentration in the range from 0.001 to 10 ng mL^{-1} with a regression equation expressed as $I = 1.86\lg c + 7.22$ ($R^2 = 0.9975$) (Fig. 5B). The limit of detection was 0.26 pg mL^{-1} at a signal-to-noise ratio of 3. The wide response range of the proposed immunosensor to PSA level was in normal and early stage of prostate cancer people, showing the potential application of this proposed method in clinical diagnosis.

Compared with those reported previously for detection of PSA, the proposed immunosensing method showed the most excellent performance in linear concentration range and the limit of detection (Table 1), which should be attributed to the loading of a large amount of MB in the conjugated DNA dendrimer for amplified immunoassay. Moreover, both the DNA-labeled antibody and the DNA dendrimer could be easily prepared, and the immunoassay procedure could be conveniently operated.

3.6. Selectivity of immunosensing method

Selectivity is of great importance to the performance of biosensors. Here, the selectivity of the immunosensing method was examined with different protein biomarkers such as AFP, CEA, NT-proBNP and SARS-CoV-2 as well as BSA (5 ng mL^{-1}) as interfering substances. As shown in Fig. 6, the immunosensor did not show significant SWV signal to these interfering proteins even at the same concentration, while target PSA led to obvious increase of the peak current. These results indicated that the prepared biosensor possessed a good selectivity for PSA detection.

3.7. Application in serum sample analysis

To evaluate the analytical reliably and application potential of the designed immunosensing method, the detection results of PSA in human serum samples were compared with those obtained with commercial electrochemiluminescent single-analyte tests after appropriate dilution. As listed in Table 2, the results for different samples showed acceptable with relative errors less than 13.3%, demonstrating good accuracy of the proposed method for clinical sample detection.

Table 2
PSA analysis in serum samples comparing with commercial ELISA kit.

Sample No.	Proposed method (ng·mL-1)	Reference method (ng·mL-1)	Relative error (%)
1	0.13	0.15	−13.3
2	0.11	0.12	−8.3
3	29.6	28.6	3.4
4	17.7	17.2	2.9
5	10.0	10.6	−5.7

4. Conclusions

A DNA dendrimer has been designed for loading of a large amount of electrochemical active molecule, methylene blue, to amplify the immunosensing signal. The DNA dendrimer can be conveniently assembled with a couple of complementary Y-shaped DNAs, and bound to immunosensor with the secondary antibody labeled with one of the complementary DNA sequences via a sandwiched immunoreaction. The three-dimensional meshy DNA dendrimer shows nearly spherical morphology, and the proposed immunosensing method for PSA detection shows excellent performance with wide detectable concentration range and low limit of detection down to sub-pg·mL^{-1} without additional amplification. The good selectivity and accuracy for serum sample analysis demonstrate the potential application of the designed DNA dendrimer in signal amplification and the proposed method in clinical diagnosis.

CRediT authorship contribution statement

Linfei Xiong: Conceptualization, Formal analysis, Investigation, Writing − original draft, Writing − review & editing. **Zhaohui Li:** Formal analysis. **Guangming Li:** Visualization. **Huangxian Ju:** Supervision, Writing − original draft, Writing − review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

We gratefully acknowledge the National Natural Science Foundation of China, China (21635005, 21827812, 21890741).

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