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PAPER

A DNA biosensor based on graphene paste electrode modified with Prussian blue and chitosan†

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A chemically modified graphene paste electrode was prepared by incorporating appropriate amounts of graphene in a paste mixture, followed by electrodepositing Prussian blue (PB) and coating chitosan on the electrode surface. The electrode was able to bind ssDNA, and gave a better voltammetric response for complement DNA than did ordinary carbon paste electrodes. The response of the electrode was characterized with respect to the paste composition, immobilization time of probe DNA on the chitosan and PB modified graphene paste electrode, and the effect of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC). The electrochemical behavior of PB assembled on the graphene paste electrode was investigated. The combination of graphene and PB can enhance the current response of the graphene paste electrode. As a consequence of DNA hybridization, a significant change in the current due to daunomycin intercalated with double-stranded DNA (ds-DNA) on the surface of the graphene paste electrode was observed.

Introduction

The Human Genome Project is providing massive amounts of genetic information that is revolutionizing the understanding and diagnosis of inherited diseases. The development of an effective DNA-sensing system with the potential for genomic sequencing, mutation detection, and pathogen identification is extremely important.[1] As an efficient method for the detection of DNA hybridization, DNA biosensors have enormous potential due to their high sensitivity, inherent simplicity and miniaturization, and low cost. Carbon electrodes are widely used in electroanalysis because of their low background current, wide potential window, chemical inertness, and suitability for various modes of sensing and detection.[2,3] Among the several forms of carbon electrodes, glassy carbon (GC) and carbon paste (CP) are the most popular carbon electrode materials.

Carbon paste, a mixture of carbon powder and a suitable liquid binder, represents one of the most frequent laboratory-made electrode materials and apparently is the most flexible substrate for chemical and biological modifications.[4–6] Moreover, the modification of carbon pastes represents a unique approach when the electrode is modified simultaneously on the surface and in the bulk.[7] Generally, the carbon powder in the carbon paste electrode is graphite. However, as far as we know, there are few reports on the use of graphene instead of graphite as the carbon powder. The recent discovery of graphene has attracted much attention because of its dimension and electronic properties. This unique nanostructured material has high surface area, excellent electrical conductivity and electron mobility at room temperature, robust mechanical properties, and flexibility.[8] Due to its special properties, there has been much interest in the biological and electrocatalytic application of graphene. Shan *et al.* constructed a graphene-based electrochemical glucose biosensor, which exhibited the potential application of graphene in biosensors.[9] Berry *et al.* fabricated a novel graphene-based live-bacterial-hybrid device and a DNA-hybridization device with excellent sensitivity.[10] Huang *et al.* simultaneously detected adenine and guanine with a graphene–COOH modified glassy carbon electrode.[11] In all these cases, the graphene-based nanocomposite film displayed an extraordinarily small electrical percolation threshold due to the high conductivity and aspect ratio of the graphene sheets.[12–18]

Prussian blue (PB), an artificial peroxidase, is a superior and selective electrocatalyst for hydrogen peroxide reduction,[19] even in the presence of oxygen.[20–23] It is an inorganic polycrystal with well-known electrochromic and electrocatalytical properties,[24] which can be applied in the construction of DNA-based biosensors. However, PB modified electrodes are disrupted after a few potential scans at neutral pH[25] which limits their use in biosensor fabrication. Adding protective films on the surface of PB may be an effective method to increase the operational stability.[23] Chitosan (Chit), a biocompatible polymer with unique structural features, possesses a primary amine at the C-2 position of the glucosamine residues. When dissolved in solvent, the Chit film with a positive charge due to NH_3^+ groups can immobilize the oligonucleotides probe, not only protecting the PB film, but also adhering to negatively charged surfaces or

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adsorbing negatively charged materials.[26] Therefore, in the presence of the water-soluble coupling reagent, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), the oligonucleotides probe with phosphate groups at the 5′ end are covalently immobilized onto the amino groups of Chit.[27]

In the present work, we constructed a novel graphene paste electrode (GPE) and intended to combine the above-mentioned benefits of graphene, PB and Chit to prepare a DNA sensor. The sensor exhibited a sensitive response for complementary DNA due to the synergistic effect of graphene, PB and Chit. The results obtained indicate that graphene could be used for the preparation of carbon paste electrodes instead of graphite. Moreover, PB assembled on the surface of a graphene paste electrode (GPE) improved the response sensitivity of DNA on the GPE. This study has opened up a new avenue for fabricating excellent electrochemical biosensors.

Experimental

Apparatus and reagents

A CHI 760C electrochemical analyzer (Shanghai Chenhua Instrument Company, China) connected to a GPE as working electrode ($\Phi = 4$mm), an Ag/AgCl reference electrode (saturated KCl) and a platinum wire auxiliary electrode, was used for electrochemical measurement. Transmission electron microscopy (TEM) was carried out using a JEM-2010 instrument (JEOL, Japan). Raman spectra were obtained with a Jobin Yvon micro-Raman spectroscope (Jobin Yvon, France). The cycle voltammetry (CV) experiments were carried out in phosphate buffered saline (PBS) solution in an electrochemical cell. All electrochemical measurements were conducted in a 10 ml cell.

PBS (0.1 M, pH 7.3) with 0.1 M KCl was used as the supporting electrolyte. Natural powdered graphite (sized $\leq$ 30 μm), chitosan, nitric acid, sulfuric acid, potassium chlorate, ethanol and hydroquinone were provided by Shanghai Chemical Reagent Company. Nujol oil was purchased from Shanghai Qinxi Chemical Technology Company. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Sigma-Aldrich company. All the chemicals used in the experiments were of analytical grade. Milli-Q18.2 MΩ water was used in all the experiments. Twenty-four-base synthetic oligonucleotides were purchased from Invitrogen Bioengineering Ltd. Company (Shanghai, China):

Probe DNA (ssDNA): 5′-PO_4-GAG CGG CGC AAC ATT TCA GGT CGA-3′

Target DNA (cDNA): 5′-TCG ACC TGA AAT GTT GCG CCG CTC-3′

Three mismatch-containing sequence (mcDNA): 5′-TCG TCC TGA AAC GTT GCG CCT CTC-3′

Noncomplementary DNA (ncDNA): 5′-GAG CGG CGC AAC ATT TCA GGT CGA-3′

Procedure

Preparation of graphene. Graphene was prepared according to a previously described method.[28] Briefly, graphite powders were first oxidized by potassium chlorate in the presence of concentrated nitric acid and sulfuric acid for 120 h. After oxidation of graphite, the mixture was added to excess water, washed with a 5% solution of HCl, and then repeatedly washed with water until the pH of the filtrate was neutral. The oxidized graphite was then suspended in a mixture of ethanol and water followed by ultrasonication for 1 h. The exfoliated graphite oxidation was reduced to graphene nanoplatelets by hydroquinone for 20 h. Finally, graphene sheets were centrifuged, washed and vacuum-dried prior to functionalization with hydroxyl and carboxylic groups. The successful synthesis of graphene was confirmed by transmission electron microscopy and Raman spectrometry (Fig. S1 and Fig. S2 in the ESI†), and the dimension of graphene was found to be under 10 nm.

Preparation of electrode. The GPE was prepared by mixing graphene and Nujol oil at different ratios.[29] The paste was carefully hand-mixed in a mortar and then packed into one end of a Teflon tube (4 mm bore, 1 mm wall thickness). The electrical contact was provided by a copper wire connected to the paste in the inner hole of the tube. The surface of the resulting paste electrode was polished on transparent paper until the surface had a shiny appearance. The electrode was rinsed carefully with water prior to each measurement.

The PB film was electrodeposited as follows:[30] the GPE was inserted in an aqueous solution consisting of 2 mM $FeCl_3$, 2 mM $K_3[Fe(CN)_6]$, 0.1 M KCl, 0.1 M HCl and a constant potential of +0.4 V (*vs.* SCE) was applied for 120 s. The electrode was then carefully washed with water and transferred into the supporting electrolyte solution containing 0.1 M KCl and 0.1 M HCl, where the electrode was cycled 20 times between 0.05 and +0.35 V at a scan rate of 40 mV s^{-1}, washed with water, and then dried with nitrogen (denoted as PB/GPE). Then 6 μl of Chit was dropped on the surface of the PB modified GPE and dried in air to form a layer of Chit membrane (denoted as Chit/PB/GPE).

DNA immobilization on the Chit/PB modified GPE

The Chit/PB/GPE was immersed in 10 mM phosphate buffer (pH 7.3) containing 2.12×10^{-5} M oligonucleotide and 0.1 M EDC for 10 h with stirring at room temperature. The oligonucleotide probe was immobilized through the formation of phosphoramidate bonds between the amino group of Chit and phosphate group of the oligonucleotides at the 5′ end, under the effect of EDC, on the surface of the modified electrode. Finally, the electrode was washed with water several times to remove the unbound DNA probes. After DNA was immobilized on the electrode, the resulting electrode was denoted as ssDNA/Chit/PB/GPE.

Blank graphene paste was prepared in the same way without PB, Chit and ssDNA.

Hybridization of DNA and electrochemical measurement

The hybridization reaction was conducted by immersing the ssDNA/Chit/PB/GPE into a hybridization solution (PBS 0.1 M, pH 7.3) containing different concentrations of DNA target for 30 min at 40 °C. After that, the electrode was rinsed three times with water to remove the non-hybridized target DNA.

The hybridized electrode was placed in 0.1 M PBS solution (pH 7.3) containing 10^{-5} M daunomycin for 5 min. Then the electrode was washed with water several times to remove the physically adsorbed molecules. Fig. 1 is a schematic illustration of the preparation procedure for DNA/Chit/PB/GPE.

The electrochemical measurement of hybridization was performed in a 10 ml electrochemical cell with a hybridized ssDNA/Chit/PB working electrode, an Ag/AgCl reference electrode and a platinum wire counter electrode. The differential pulse voltammetry (DPV) measurements were conducted from 0.6 to 0.1 V (*versus* Ag/AgCl) in 0.1 M phosphate buffer (pH 7.3). Under these conditions, a peak current related to the concentration of DNA at about +0.368 V was taken as the electrochemical measurement signal.

Results and discussion

Optimization of detection conditions

Choice of the paste composition. The conductivity of the GPE material affects the electrochemical analytical signals of GPE. This effect could also be expressed using typical electrochemical data. Fig. 2 shows the effect of the paste composition on the peak potential separation (ΔE_p) and peak current (I_p) of blank GPE in 10 mM $K_3Fe(CN)_6$: $K_4Fe(CN)_6$, between −0.6 and 1.0 V at a scan rate of 100 mV s^{-1}. Graphene paste electrodes containing 7, 9, 12, 15, 17, 20, 22, 24 and 26% Nujol oil were employed.

From Fig. 2, it can be seen that when a Nujol oil : graphene ratio of 7/93 (w/w) was used, in which the composition of the paste is similar to the dry carbon powder, the response of the GPE showed the smallest separation of the peak potentials (ΔE_p) and highest peak current (I_p) in $K_3Fe(CN)_6$: $K_4Fe(CN)_6$ solution in this experimental case. Moreover, the separation of the peak potentials (ΔE_p) became larger and the peak current (I_p) became smaller with an increasing amount of Nujol oil from 7% to 26%. This possibly contributed to the increased resistance of the GPE when the amount of Nujol oil in the paste increased while the amount of graphene powder decreased in the composition of the GPE.

Taking into account that the high surface area of graphene could allow us to prepare stable and robust paste electrodes using higher amounts of Nujol oil, we investigated a 12 : 88 (w/w) Nujol oil : graphene composition to prepare the GPE.

Immobilization time of probe DNA on the Chit/PB/GPE and the effect of EDC

The sensitivity of the DNA hybridization assay was directly related to the surface coverage of DNA probes on the electrode,

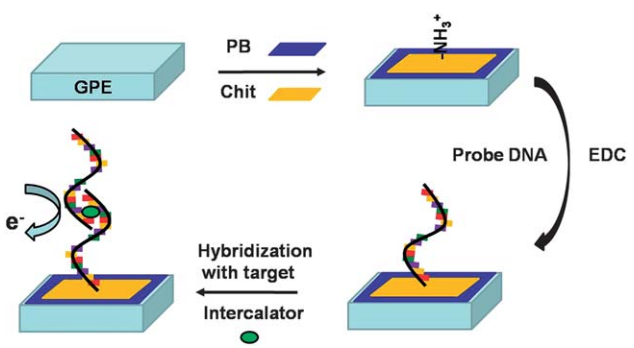

Fig. 1 The experimental scheme of the modified electrode.

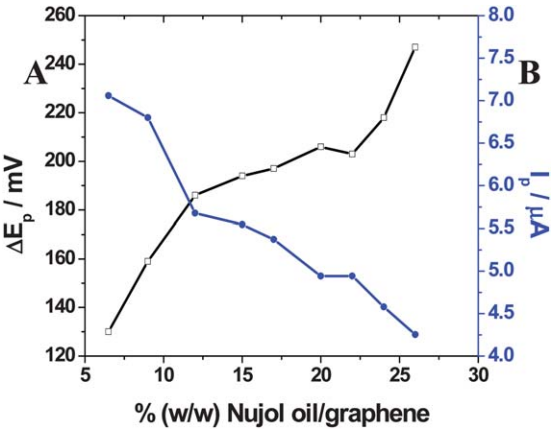

Fig. 2 Effect of the paste composition on (A) peak potential separation (ΔE_p) and (B) peak current (I_p) of 10 mM $K_3Fe(CN)_6$: $K_4Fe(CN)_6$. Compositions: 7 : 93, 9 : 91, 12 : 88, 15 : 85, 17 : 83, 20 : 80, 22 : 78, 24 : 76 and 26 : 74 (w/w) % (Nujol oil : graphene). Scan rate: 100 mV s^{-1}.

the behavior of DNA hybridization can be affected by daunomycin. At room temperature, the differential pulse voltammetry (DPV) responses of the electrode for DNA increased with an increase in the immobilization time of the probe DNA in Chit film on the PB modified graphene paste electrode and then tended to reach constant values after 10 h (Fig. 3), which showed a saturated binding between the probe DNA and Chit on the electrode surface. And we can see that the DPV responses of the electrode for DNA are significantly larger with the help of EDC than that without it. Therefore, 10 h of immobilization time with the help of EDC was selected for the electrode.

The effect of hybridization temperature and incubation time

The effect of the hybridization temperature on the response signal was investigated from 25 to 65 °C. The peak current reached a maximum value at a hybridization temperature of 40 °C, indicating that the highest hybridization efficiency was obtained at 40 °C. As a result, 40 °C was selected in the subsequent work.

The effect of incubation time between ssDNA/Chit/PB/GPE and the target DNA was also tested. The hybridized-electrode

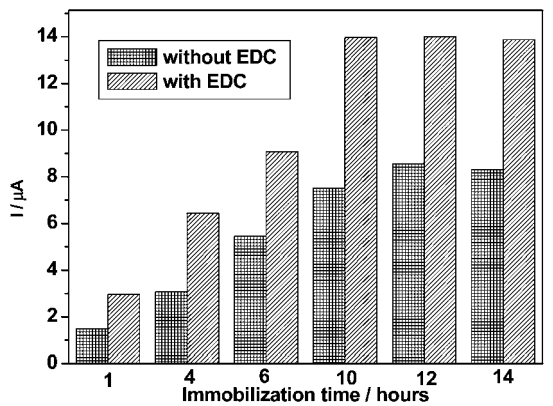

Fig. 3 Effects of immobilization time on DPV responses of DNA at the modified electrode with and without EDC.

was incubated in PBS (pH 7.3) containing 2.12×10^{-8} M cDNA, and the peak current of DNA increased with an increasing in incubation time, reaching a current plateau when the incubation time reached 25 min. Therefore, the optimum incubation time was 30 min.

Electrochemistry of modified electrodes in PBS solution

Fig. 4 shows the cyclic voltammograms of the bare GPE, PB/GPE, Chit/PB/GPE and DNA/Chit/PB/GPE in 0.1 M PBS (pH = 7.3) at a scan rate of 50 mV s^{-1}. It can be seen that neither oxidation nor reduction took place on the bare GPE (curve a in Fig. 4). After the Prussian blue layer was modified on the graphene paste electrode, two well developed stable redox waves ($E_{pa1} = 0.20$ V, $E_{pc1} = -0.06$ V, $E_{pa2} = 0.91$ V, $E_{pc2} = 0.75$ V, curve b in Fig. 4) were observed when the potential was cycled between −0.6 and 1.0 V. The typical two pairs of redox waves showing the oxidation of Prussian blue to Prussian green as well as the reduction to Prussian white are the response characterization of Prussian blue for all the sensors,[24] indicating that PB was successfully electrodeposited on the surface of the paste. However, for the Chit/PB/GPE, when a potential sweep was cycled between −0.6 V and 1.0 V, the currents of two peaks were decreased because Chit was not oxidized or reduced (curve c in Fig. 4). After ssDNA/Chit/PB/GPE hybridized cDNA with incubated daunomycin, the redox peak of guanine was 0.386 V and 0.154 V (curve d in Fig. 4).

Primary application

The most common electrochemical strategies for detecting the hybridization of DNA rely on interacting electroactive substances such as a groove binder or intercalating organic compounds (*e.g.*, daunomycin) that interact in different ways with ssDNA or dsDNA. The electrochemical detection of DNA *via* an interacting electroactive substance is an attractive approach for oligonucleotides hybridization measurements because the target DNA does not need to be chemically modified.[31]

Daunomycin is a well-known electroactive hybridization indicator of DNA biosensor.[32,33] The interaction of daunomycin with DNA has been deeply studied by Chaires and co-workers.[34–37] It appears that daunomycin intercalates into duplex DNA with preferential binding to G–C base pairs.

The effect of PB on the response of the modified electrode was investigated by comparing the DPV response of DNA/Chit/GPE and DNA/Chit/PB/GPE (Fig. 5). From Fig. 5, the DPV response of DNA/Chit/GPE (curve a) is shown to be significantly smaller than that of DNA/Chit/PB/GPE (curve b) when both electrodes are hybridized in 2.12×10^{-8} M cDNA solution and 1×10^{-5} M daunomycin. The results confirmed that the use of PB can improve the performance of the modified electrode. This possibly contributed to the synergism of PB and graphene in the paste. In addition, the potential of DNA/Chit/GPE (curve a) was more positive than that of DNA/Chit/PB/GPE (curve b), the reason may be that PB, as an electrochemical inorganic mediator, results in a decrease of the applied potential and the consequent avoidance of many electrochemical interferences.[38]

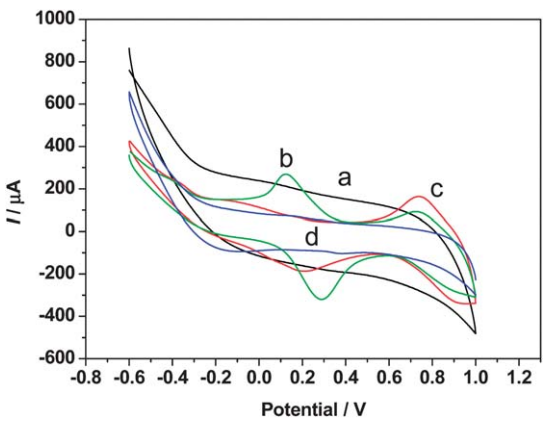

Fig. 4 Cyclic voltammograms of different modified electrodes in 0.1 M PBS (pH 7.3). (a) bare GPE; (b) PB/GPE; (c) Chit/PB/GPE; (d) DNA/Chit/PB/GPE. Scan rate: 50 mV s^{-1}.

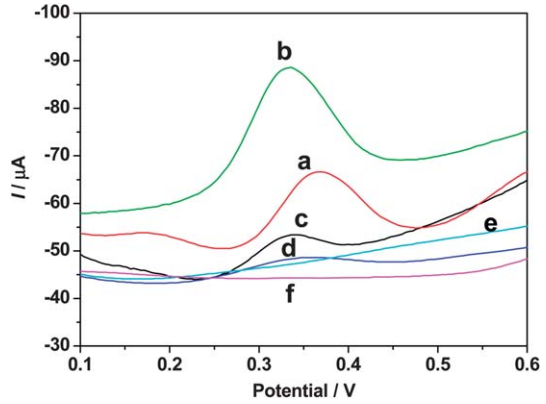

Fig. 5 The DPV response of DNA/Chit/GPE (a) hybridized in 2.12×10^{-8} M cDNA solution; the DPV response of DNA/Chit/PB/GPE; (b) hybridized in 2.12×10^{-8} M cDNA solution; (c) hybridized in 2.12×10^{-8} M mcDNA solution; (d) hybridized in 2.12×10^{-8} M ncDNA solution and incubated in 1.0×10^{-5} M daunomycin; (e) ssDNA/Chit/PB/GPE hybridized cDNA solution without daunomycin; (f) ssDNA/Chit/PB/GPE hybridized without cDNA, but incubated with daunomycin (0.1 M pH 7.3 PBS).

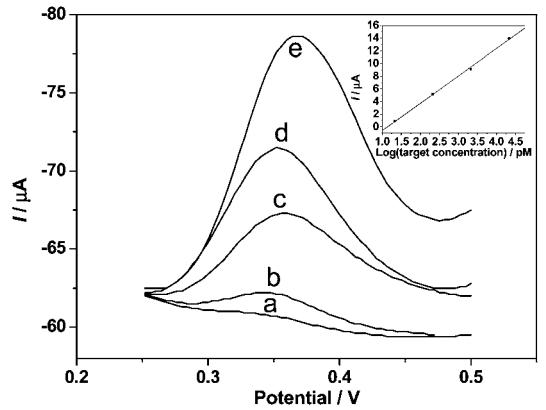

Fig. 6 DPV response of the ssDNA/Chit/PB/GPE after hybridization with increasing concentration (M) of complementary target DNA (a) 0; (b) 2.12×10^{-11}; (c) 2.12×10^{-10}; (d) 2.12×10^{-9}; (e) 2.12×10^{-8}. Insert: the calibration curve of the biosensor.

Table 1 Comparison of response of different DNA sensors

Electrode	Modifier	Method	pH	Detection limit (mol dm^{-3})	Dynamic range (mol dm^{-3})	γ^2	References
Glassy carbon	Graphene-COOH	DPV	4.5	5×10^{-8}	5×10^{-7}–2×10^{-4}	0.9941	11
Glassy carbon	Carbon nanotubes	DPV	4.8	1.6×10^{-8}	3.12×10^{-7}–6.25×10^{-6}	0.968	39
Glassy carbon	SWNTs	DPV	7.0	2×10^{-8}	4×10^{-8}–11×10^{-8}	0.9906	40
Glassy carbon	MWCNTs–COOH	DPV	6.5	3.81×10^{-11}	1.6×10^{-9}–4.8×10^{-8}	0.9898	41
SiO_2	Aligned carbon nanotube	DPV	9.6	2×10^{-11}	5×10^{-10}–5×10^{-7}	0.98	42
Graphene paste	Prussian blue–chitosan	DPV	7.3	1.58×10^{-11}	2.12×10^{-11}–2.12×10^{-8}	0.9992	This work

The effect of different sorts of DNA on the process of hybridization was investigated by comparing the DPV response current of ssDNA/Chit/PB/GPE hybridized with cDNA, mcDNA and ncDNA, respectively. Fig. 5 displays the comparison of DPV response of the ssDNA/Chit/PB/GPE in different cases. It can be seen that there is nearly no current response for the DNA sensor hybridized without cDNA, but incubated with daunomycin (curve f), indicating that the presence of non-specific adsorption of daunomycin doesn't influence the result of this experiment. When the electrode was hybridized in 2.12×10^{-8} M ncDNA solution, it exhibited a very low response current (curve d). For the electrode hybridized with cDNA but not incubated in daunomycin a lower current response was also shown (curve e). However, when the modified electrode was treated with 2.12×10^{-8} M cDNA solution and incubated in 10^{-5} M daunomycin indicator solution, the response current was greatly enhanced, demonstrating that ssDNA/Chit/PB/GPE can be used to detect target DNA, which was complementary with its DNA probe (curve b). For a three-mismatched DNA, the electrode showed a smaller response (curve c). The amperometric data confirmed that the proposed method can successfully detect target DNA with high specificity and sensitivity.

The analytical performance of the DNA biosensor was verified by using the immobilized probe to hybridize the different concentrations of the complementary sequence according to the described procedure. The DPV response of the ssDNA/Chit/PB/GPE increased with an increase in the concentration of complementary target DNA (Fig. 6). The peak currents of the ssDNA/Chit/PB/GPE are linear with the logarithmic value of the cDNA sequence concentration from 2.12×10^{-8} to 2.12×10^{-11} M. The regression equation was $I_p/\mu A = 4.32 \lg C/10^{-12}\,M - 4.91$ (C was the concentration of the complementary sequence, 10^{-12} M; I_p was the peak current of the electrode, μA; the number of points for calculating the equation was 4), and the regression coefficient (R) of the linear curve was 0.9992. The detection limit, taken as the concentration that gave a signal equal to three times the standard deviation of the blank signal, of ssDNA/Chit/PB/GPE for its complementary DNA sequence was 1.58×10^{-11} M ($n = 11$), which is comparable with the values reported by other research groups (Table 1).

Electrode reproducibility and stability

The ssDNA/Chit/PB/GPE can be used for the determination of DNA by DPV. The repeatability of the DNA biosensor was assessed by repeated measurements in 0.1 M PBS (pH 7.3); the standard deviation was 2.83% ($n = 4$). When stored in 0.1 M PBS (pH 7.3) at 4 °C for more than one week, the response current of the DNA biosensor slightly decreased.

Conclusions

The direct electrochemical behavior of single-stranded DNA (ssDNA) on chitosan and Prussian blue modified graphene paste electrodes was carefully studied. Graphene, used as the carbon paste material for the electrode instead of graphite, can greatly improve the sensitivity of the biosensor. The presence of a Prussian blue layer on the surface of the GPE showed good electrochemical properties, the chitosan film protected the stability of the Prussian blue film significantly. The established DNA biosensor with high selectivity can discriminate completely complementary target sequence, three-base-mismatched sequence and noncomplementary target sequence. In summary, the electrochemical properties of graphene paste electrodes and Prussian blue can offer a new approach for developing novel types of highly sensitive and stable electrochemical biosensors.

Acknowledgements

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