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PAPER

A cyclodextrin host–guest recognition approach to a label-free electrochemical DNA hybridization biosensor

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A novel label-free electrochemical DNA hybridization biosensor using a β-cyclodextrin/poly(*N*-acetylaniline)/carbon nanotube composite modified screen printed electrode (CD/PNAANI/CNT/SPE) has been developed. The proposed DNA hybridization biosensor relies on the intrinsic oxidation signals of guanine (**G**) and adenine (**A**) from single-stranded DNA entered into the cyclodextrin (CD) cavity. Due to the binding of **G** and **A** bases to complementary cytosine and thymine bases in dsDNA, the signals obtained for ssDNA were much higher than that of dsDNA. The synergistic effect of the multi-walled carbon nanotubes provides a significantly enhanced voltammetric signal, and the CD encapsulation effect makes anodic peaks of **G** and **A** shift to less positive potentials than that at the bare SPE. The peak heights of **G** and **A** signals are dependent on both the number of the respective bases in oligonucleotides and the concentration of the target DNA sequences. Hybridization of complementary strands was monitored through the measurements of oxidation signal of purine bases, which enabled the detection of target sequences from 0.01 to 1.02 nmol μl^{-1} with the detection limit of target DNA as low as 5.0 pmol μl^{-1} ($S/N = 3$). Implementation of label-free and homogeneous electrochemical hybridization detection constitutes an important step toward low-cost, simple, highly sensitive and accurate DNA assay. Discrimination between complementary, noncomplementary, and two-base mismatch targets was easily accomplished using the proposed electrode.

1. Introduction

The detection of DNA is currently an area of tremendous interest as it plays an important role in disease diagnostics, epidemic prevention, environmental protection, clinical and pharmaceutical applications.[1] Different strategies including optical,[2] surface plasmon resonance,[3] quartz crystal microbalance[4] and electrochemical techniques have been well established for DNA detection among which electrochemical transducers offer great advantages such as simplicity, high sensitivity and low-cost for converting DNA hybridization events into an analytical electronic signal.[5]

In general, strategies for detection of DNA hybridization events between DNA probes and their complementary ones are classified into two main groups; direct and indirect strategies. The direct (label-free) detection protocol relies either on the intrinsic redox-active properties of DNA bases or a change of electrical properties of an electrode interface upon hybridization, while the indirect detection protocol is based on the incorporation of an electroactive indicator.[6–10]

Until now, a variety of electrochemical approaches for detecting DNA hybridization have been reported. In most of the DNA hybridization assays, the hybridization reaction was performed with the DNA probe immobilized on the solid support to form duplex DNA on the surface. In this approach, external labels such as intercalators, metal complexes or organic dyes are used as a reporter for detection of hybridization events. In one example, cyclic voltammetry (CV) and fluorescence spectroscopy were used to investigate the interaction of some manganese complexes as an intercalator and hybridization indicator with salmon sperm DNA.[11]

There has been a considerable interest in the label-free electrochemical detection of DNA hybridization. These protocols greatly simplify DNA hybridization assays as they offer an instantaneous detection of the DNA duplex formation, and are free from indicator addition/association/detection steps. There are several comprehensive reviews that have addressed the recent advances in the development of label-free DNA hybridization biosensors.[5,12] Typical label-free transduction methods rely on monitoring the oxidation current of **G** and **A**, the most redox active nitrogenous bases in DNA. Wang's group has developed a multi-walled carbon nanotubes modified glassy carbon electrode (GCE) for the direct electrochemical detection of DNA based on enhanced **G** oxidation signal.[13] Ozsoz *et al.* immobilized an inosine-substituted (**G**-free) probe ss-DNA onto a disposable carbon graphite electrode, and the detection of hybrid formation was performed by using the appearance of the **G** oxidation signal of the target DNA (related to the catechol-*O*-methyl transferase gene) in conjunction with DPV analysis.[14] In another label-free

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approach, Erdem *et al.*[15] have developed a sensitive and low cost DNA biosensor based on a CNT modified disposable pyrolytic graphite electrode (PGE). Wang *et al.*[16] reported a study by using DPV and FTIR spectroelectrochemistry based on the oxidation signals of **G** and **A** at a glassy carbon electrode. In another study, Sun *et al.* have reported an ionic liquid modified carbon paste electrode for the detection of nucleic acids based on the oxidation of **G** residue on the ssDNA.[17] Karadeniz *et al.* have also developed a label-free DNA hybridization by using CNT modified SPEs for the electrochemical detection of DNA hybridization related to specific sequences on HBV.[18]

Carbon nanotubes (CNTs) have stimulated increasing interest in the fabrication of electrochemical sensors due to their unique combination of excellent mechanical, electrical and electrochemical properties.[19] Many applications of carbon nanotubes in electrochemical sensors for different biomolecules have been reported.[20] Recently, the modification of electrochemical sensors with carbon nanotubes (CNTs) has attracted considerable attention in the field of DNA sensing technology, since surface-confined CNT can dramatically enhance the electrochemical reactivity of many important biomolecules including DNA.

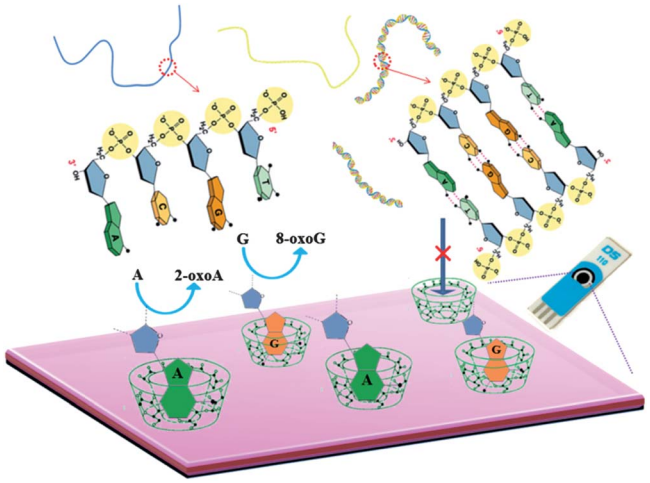
Cyclodextrins (CDs) are oligosaccharides consisting of six, seven, or eight glucose units (named α-, β-, or γ-CD, respectively) which present a toroidal form with a hydrophobic inner cavity and a hydrophilic outer side.[21] Natural and chemically modified CDs can be viewed as molecular receptors. They can accommodate a wide variety of organic, inorganic, as well as biological guest molecules to form stable host–guest inclusion complexes or nanostructure supramolecular assemblies in their hydrophobic cavity, showing high molecular selectivity and enantioselectivity.[22] Recently, Carlstedt *et al.* have reported that β-CD can be used to decompact DNA–surfactant complexes.[23] CDs are resistant to degradation by human enzymes and do not produce an immune response in mammals so they are of interest in therapeutical applications.

We have recently used a CD/PNAANI/CNT composite modified carbon paste electrode for the simultaneous quantification of serotonin and dopamine.[24] Oxidation of **G** and **A** bases occurs at highly positive potentials on bare electrodes which is associated with high background signals and compromises the sensitivity of the biosensor. In this study we report a β-CD/PNAANI/CNT composite modified screen printed graphite electrode for the quantification of DNA hybridization events related to hepatitis B virus using the oxidation signals of **G** and **A** (Scheme 1). The proposed DNA hybridization biosensor is based on competitive hybridization in an homogeneous solution phase with high hybridization efficiency and detection using the host–guest recognition technique by CV and differential pulse voltammetry (DPV). The proposed electrode benefits from two distinct functional materials: (i) the electrocatalytic activity of CNT, and (ii) the cumulative effect of CD. These features in combination with the advantages of the SPE provided very good sensitivity and selectivity for the electrode.

2. Experimental

2.1. Apparatus

Voltammetric studies were accomplished using a Metrohm electroanalyzer (Model 757 VA Computrace). A disposable screen-printed electrode consisting of a carbon working electrode, a carbon counter electrode, and a silver pseudo-reference electrode printed on a substrate was purchased from DropSens for electrochemical measurements. A cable connector (DRP-BICAC connector, DropSens) operates as an interface between the disposable screen-printed electrode and the Metrohm electroanalyzer. A Metrohm 780 pH meter was used for pH measurements.



Scheme 1 Schematic representation of the inclusion complex formation between β-cyclodextrin and **G/A** nucleobases in ssDNA as guest molecules.

2.2. Materials

N-Acetylaniline (Merck) was used after recrystallization from ethanol. Lithium perchlorate and β-CD were obtained from Fluka and the multiwalled carbon nanotube (MWNT) (OD = 10–30 nm, ID = 5–10 nm, length = 0.5–500 μM, +95%) was obtained from Aldrich. Double-stranded calf-thymus DNA (lyophilized powder) was purchased from Sigma.

Four 18-base oligonucleotides related to the hepatitis B virus (HBV) were purchased from MWG Biotech (Germany), with the following base sequences:

Probe sequence (S1): 5′-CGA CAG TGG TCC CAA AGA-3′

Complementary target sequence (S2): 5′-TCT TTG GGA CCA CTG TCG-3′

Two-base mismatch (S3): 5′-TCT T***C***G GGA CC***G*** CTG TCG-3′.

Noncomplementary (S4): 5′- CAG CGA TGG AGA TCC CAA -3′

The underlined bases in S3 indicate those mismatched with S1. All oligonucleotide stock solutions (100 pmol μl^{-1}) were prepared in phosphate buffer solution (10 mM PBS, 1mM EDTA at pH 7.5) and were stored at 4 °C. Calf thymus double-stranded DNA (dsDNA) stock solution with a concentration of 0.2 mg ml^{-1} was prepared by dissolving an appropriate amount ot dsDNA in deionized water containing 1.0 mM EDTA adjusted to pH 7.4. More diluted solutions of DNA were prepared with 0.1 M PBS containing 20 mM NaCl (pH 7.4) according to the hybridization protocol.

All other chemicals were of analytical reagent grade and were used without further purification. All experiments were carried

out at room temperature except otherwise mentioned. Deionized ultra-pure water was used for preparing the solutions.

2.3. Procedures

2.3.1. Preparation of the CD/PNAANI/CNT modified screen printed electrodes. The pristine CNT was purified and activated before use. For this purpose, the CNT was stirred in HNO_3 (2 M) for 24 h and then filtered and washed several times with deionized water for complete removal of nitric acid. The resultant CNT was then dried at 80 °C in an oven overnight. This treatment produced oxygen-containing moieties (for example carboxylic acid groups) at the open ends of the nanotubes which make it dispersible in aqueous media. CNT black suspension was prepared by dispersing 1.0 mg CNTs in 1.0 ml deionized water with the aid of ultrasonic agitation. Then, 30 μl of the black suspension was cast on the screen printed electrode surface and air-dried at room temperature. After solvent evaporation a uniform CNT film was formed. The PNAANI film electrode was prepared by electrodeposition of PNAANI in a 0.1 M *N*-acetylaniline and 1 M $HClO_4$ solution. The electrode potential at first was held at a constant voltage of 1.0 V *versus* Ag/AgCl for one minute, and then swept from −0.20 V to 1.0 V for 20 cycles at a scan rate of 100 mV s^{-1}. In the process of electrolysis, an excellent cohesive brown film was formed on the electrode surface.

The CD modified electrode was fabricated by electrooxidation of the PNAANI/CNT electrodes in a DMSO solution of 0.05 M β-CD and 0.1 M $LiClO_4$. The electrooxidation was carried out at a constant potential of 1.2 V for 10 min.

2.3.2. Denaturation of dsDNA. Single-stranded calf thymus DNA (ssDNA) was prepared by thermal denaturation of commercial calf thymus dsDNA during which the hydrogen bonds were ruptured. A solution of 0.2 mg ml^{-1} dsDNA containing 1.0 mM EDTA was heated at a high temperature of 95 °C for 20 min, followed by rapid cooling of the solution for 10 min in an ice bath.

2.3.3. Solution-phase hybridization. For the DNA hybridization reaction, 250 μl of ssDNA probe (1 nmol) and 250 μl of the desired concentration of complementary or two-base mismatched DNA were added into a tube and the mixture was subjected to incubation for 10 min at 37 °C with gentle mixing.

2.3.4. Electrochemical studies on the interaction between CD and ssDNA. **A** and **G** from ssDNA penetrate into the CD cavity, while the same bases from dsDNA could not penetrate into the CD cavity (due to binding of **G** to cytosine (**C**) and **A** to thymine (**T**) *via* the famous Watson–Crick base-pairing in dsDNA). The oxidation signals of **A** and **G**, included in the CD cavity were measured by using CV or DPV in PBS (pH 7.4). First of all, the CD/PNAANI/CNT modified SPE was activated by means of cyclic voltammetric sweeping from 0 to 1.1 V in PBS until the voltammograms were stable (about 10 cycles). After that, the desired volume of standard DNA solution was added by micropipette on top of the working electrode surface. For the CV determination, the preconcentration of the analyte at the CD-modified electrode was performed for a given period of time at the open circuit. Then cyclic voltammograms were recorded using a scan rate of 100 m Vs^{-1}. For repetitive measurements, the CD-modified electrode underwent successive cyclic sweeping between 0 to 1.1 V in the blank phosphate buffer (pH 7.4) to give a fresh electrode surface. An electrode surface prepared according to the above mentioned procedure could be used for at least 4 independent analyses. The best parameters for DPV on the CD-modified electrode were: accumulation time 120 s; pulse amplitude 0.05 V, scan rate 0.015 V s^{-1} and pulse time 0.04 s.

After each measurement the electrode surface was regenerated by immersing in 0.1 M PBS for a specified time.

3. Results and discussion

3.1. Electrochemical characterization of β-CD/PNAANI/CNT modified SPE

The proposed DNA hybridization biosensor relied on the oxidation signals of **G** and **A**. The detection method involves monitoring the intrinsic signals of **G** and **A** from ssDNA by CV or DPV, which is greatly enhanced at the CD/PNAANI/CNT modified SPE. The decrease in the magnitude of the voltammetric peaks of **G** and **A** thus reflected the extent of the hybrid formation.

The electrochemical behavior of **G** and **A** within ssDNA and dsDNA at bare and modified screen printed electrodes was investigated by CV. Fig. 1 shows the cyclic voltammograms of **G** and **A** residues within ssDNA in PBS (0.1 M) on the CNT modified (Fig. 1, curve **a**), CD/PNAANI modified (Fig. 1, curve **b**) and CD/PNAANI/CNT modified (Fig. 1, curve **c**) SPE (background currents are subtracted for simplicity). The concentration of ssDNA was kept constant at 0.5 nmol μl^{-1}. Purine bases within ssDNA exhibit negligible electrochemical activity on the bare SPE. The oxidation peak current of **G** and **A** on the CNT modified electrode was much higher than that on the bare electrode but their peak potentials remained almost unchanged (Fig. 1, curve **a**). The result is consistent with the reported literature,[13] in which they attributed the enhanced electrochemical response of free **G** at the CNT modified electrode

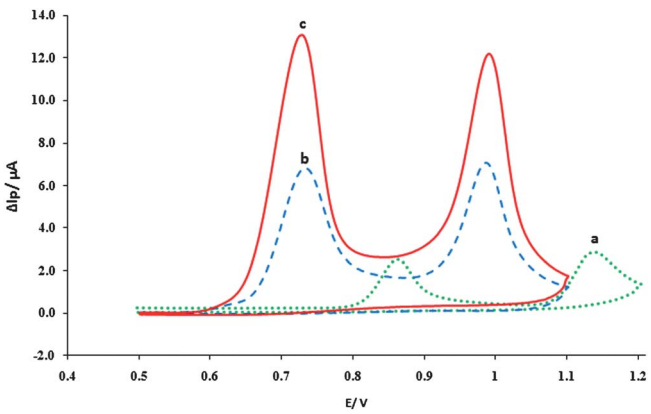


Fig. 1 Cyclic voltammograms of 0.5 nmol μl^{-1} ssDNA in pH 7.4 phosphate buffer (0.05 M) on the MWNT modified (**a**, round dots), CD/PNAANI modified (**b**, dashed) and CD/PNAANI/CNT modified (**c**, solid) screen printed electrode (background currents are subtracted for simplicity).

to the interfacial accumulation effect rather than to an accelerated electron transfer. However, the signal was considerably diminished after electrodeposition of PNAANI film on the CNT modified electrode (*i.e.* PNAANI/CNT modified SPE). A substantial enhancement of the **G** and **A** peaks is observed at the CD/PNAANI modified SPE along with a considerable change in the peak potential which is fully attributed to the CD encapsulation effect (Fig. 1, curve **b**). Based on the combination of two kinds of functional materials (MWNTs and β-CD), the CD/PNAA/CNTs modified SPE (Fig. 1, curve **c**) exhibits the best analytical performance in the determination of ssDNA. Oxidation peaks of ssDNA at the CD/PNAANI/CNT modified SPE shift considerably in the negative direction, indicating that ssDNA can be oxidized more easily at the proposed modified electrode. This appearance indicates that CNT catalyze the oxidation of **G** and **A** residues and CD accumulate and preconcentrate ssDNA at the electrode surface.

The amplified **G** and **A** signals at the CD/PNAANI/CNT modified screen printed electrode are exploited for measurements of nucleic acids and for label free detection of DNA hybridization. The signals obtained for ssDNA were much higher than the ones obtained for dsDNA. Due to the binding of **G** and **A** bases to complementary **C** and **T** bases in dsDNA, **G** and **A** were prevented from inclusion into the CD cavity and were only partially available for oxidation, so the peak current observed for them diminished considerably.

The consistency of the proposed biosensor was tested in the presence of calf thymus ds-DNA and the thermally denatured one (ss-DNA). Fig. 2 shows the corresponding CVs for calf thymus DNA by measuring **G/A** oxidation signals. A negligible response with a hump peak was observed at about +1.0 V for dsDNA (Fig. 2, curve **b**) which could be attributed to the non-catalyzed oxidation of purine bases in the dsDNA scaffold. After thermal denaturation of the corresponding dsDNA to ssDNA, a pair of oxidation signals was observed at oxidation potentials of **G** and **A** (Fig. 2, curve **c**). In this case, 30 cycles were applied for the electrodeposition of PNAANI film and hence the background current for the electrode (Fig. 2, curve **a**) is higher compared with the other electrodes.

A two-base mismatch containing synthetic oligonucleotide was also hybridized with the probe sequence and the oxidation peaks of **G** and **A** were studied (Fig. 3). This clear difference between the complementary (S2) (Fig. 3, curve **a**) and the two-base mismatch (S3) sequence (Fig. 3, curve **b**) (in an equimolar concentration with S1) indicated that complete hybridization was not accomplished with a two-base mismatch sequence. When the electrode interacted with the noncomplementary sequence (S4) in an equimolar concentration with the complementary one (Fig. 3, curve **d**), the signal obtained was almost twice that of the signal obtained with the probe sequence (S1) (Fig. 3, curve **c**). The increase in the amplitude of the voltammetric oxidation signals of **G** and **A** reflect the true extent of hybrid formation, *i.e* zero percent hybridization between noncomplementary and probe sequence.

Additional insights into the surface accumulation and stability of the CD modified electrode were obtained in medium-exchange experiments. In adsorptive transfer stripping (medium exchange) voltammetry, ssDNA is first adsorbed at the CD modified electrode, then the electrode is washed and transferred (with the adsorbed layer) in the medium not containing DNA, and voltammetric analysis is performed in this medium. The exchange of solutions between the preconcentration and measurement steps offers the advantage of measuring the surface-accumulated species without interference from diffusing analytes. To do so, the electrode was removed from the sample solution, rinsed thoroughly with water, and reimmersed in the supporting electrolyte solution. A medium exchange experiment for CD/PNAANI/CNT electrode resulted in a peak that was about 70% of the height of the original peak, while no peak was recorded after medium exchange using a PNAANI/CNT modified electrode. These data indicate that the purine bases have a significantly strong affinity towards the CD cavity. The electrochemical behavior of **G** and **A** residues in ssDNA at a CD modified electrode resemble those of free **G** and **A**, which has been extensively investigated in our previous study.[25]

DNA hybridization has been studied extensively in bulk solution and on a variety of solid supports using a number of

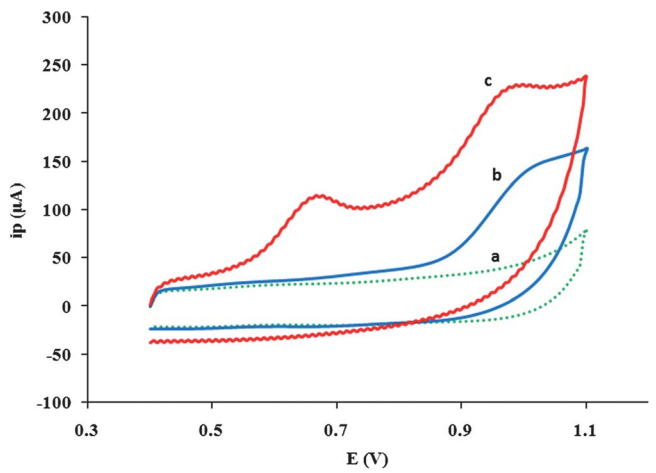


Fig. 2 CVs of the calf thymus DNA prior to (**b**) and after (**c**) thermal denaturation. Curve (**a**) indicates the background current of PNAANI film electrode. All voltammetric experiments were performed in 0.1 M phosphate buffer, 0.2 M NaCl (pH 7.4) after 5 min accumulation at CD/PNAANI/CNT modified electrode. The scan rate was 100 mVs^{-1}.

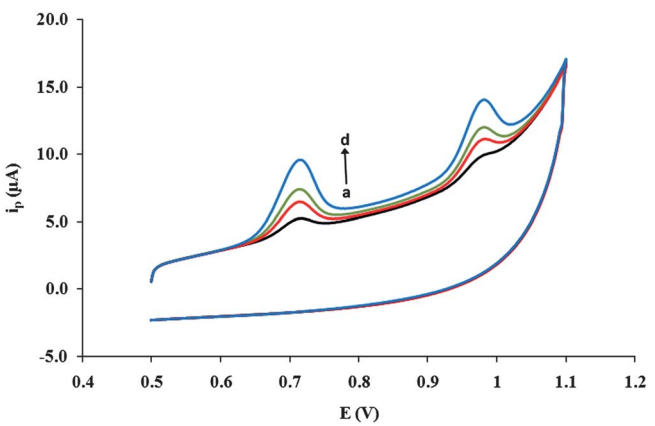


Fig. 3 A set of CVs of the probe sequence (curve **c**) in the presence of equimolar concentrations of complimentary (curve **a**), two-base mismatch (curve **b**) and noncomplementary (curve **d**) sequences.

different experimental techniques and conditions. In most of the DNA hybridization assays, the hybridization reaction was performed with the DNA probe immobilized on the solid support to form duplex DNA on the surface. In the present work, the hybridization reaction is performed in the solution phase. The efficiency and detecting sensitivity of solid phase hybridization reactions tends to be lower than that of solution phase hybridization since not all sequences are available for hybridization. In solution phase hybridization, both the probe sequence and the target are free to interact in the reaction mixture, which speeds the rate at which duplex molecules are formed.[26] Furthermore, the employed liquid DNA hybridization reaction in the present work also simplifies the experimental procedure and avoids many separation and washing steps. The only drawback we have found with it is that, there is no way to separate single stranded DNA's remaining unhybridized during the hybridization process which could be easily overcome with the background subtraction.

3.2. Optimization of experimental parameters

Possible factors that may contribute to the enhancement of the **G** and **A** voltammetric signals at the CD/PNAANI/CNT modified electrode have been examined and optimized.

3.2.1. Effect of scan rate and solution pH. The effect of scan rates on the electrochemical response of ssDNA adsorbed at the CD modified electrode was studied. With an increase in the scan rate, the oxidation peak current of **G** residues in ssDNA increased gradually and then tended to level off, and the oxidation peak potential shifted towards more positive positions. Results showed that the anodic peak current (I_{pa}) was directly proportional to both the scan rate ($R^2 = 0.992$) and the square root of the scan rate ($R^2 = 0.991$) in the range of 10–200 mV s^{-1} (Fig. 4). The phenomenon indicated that the electro-oxidation process of **G** residues in ssDNA at the CD modified electrode was controlled not only by the diffusion, but also the adsorption on the electrode surface.

The effect of pH of PBS on the response of **G** residue in adsorbed ssDNA was obvious due to the participation of protons in the electrode process. pH 7.4 was chosen because the maximum peak current change (before and after addition of ssDNA) was obtained.

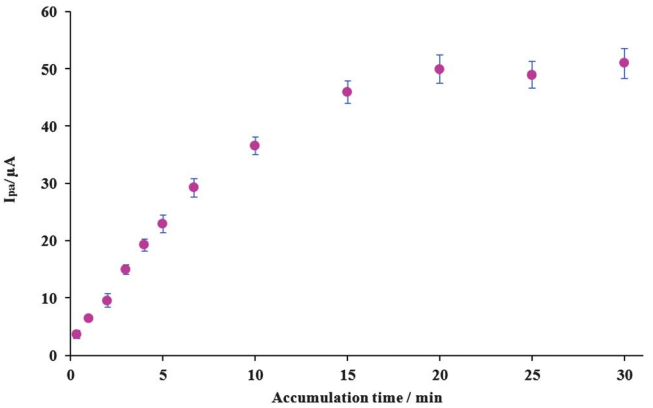


Fig. 5 Influence of accumulation time on the oxidation peak current of guanine residues in ssDNA.

3.2.2. Effect of accumulation time and potential. The results revealed that the accumulation potential did not affect the oxidation peak currents, and hence open-circuit accumulation was performed in this study. The influence of accumulation time on the oxidation peak currents at the CD/PNAANI/CNT modified SPE was investigated from 0 to 30 min (Fig. 5).

The oxidation peak currents increased sharply within the first 400 s, and then continued with a moderate slope up to 900 s. Considering sensitivity, speed and an extended dynamic range, an accumulation time of 400 s was employed. It should be mentioned that at higher accumulation times even lower concentrations can be detected.

3.3. Analytical application

The use of the amplified **G** and **A** responses at a CD/PNAANI/CNT modified electrode for DNA hybridization detection is illustrated for detecting ssDNA segments related to the HBV. The voltammetric signals obtained with the ssDNA were higher than the ones obtained with the hybridized dsDNA because of available purine bases for oxidation.

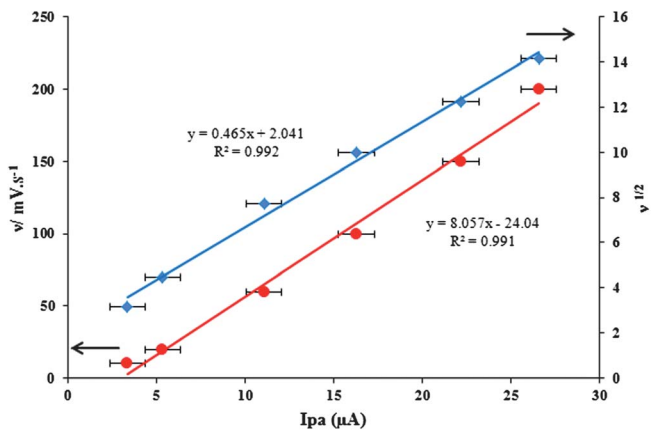


Fig. 4 Proportionality of guanine oxidation peak current with scan rate, v ($R^2 = 0.9911$) and square root of the scan rate, $v^{1/2}$ ($R^2 = 0.9923$) ($n = 4$).

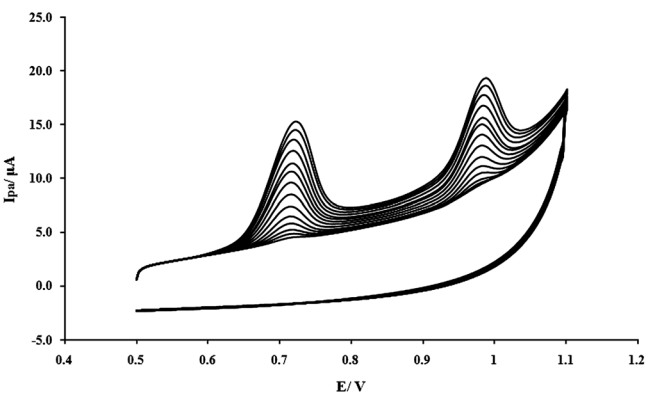


Fig. 6 CVs at different concentrations of ssDNA in optimum conditions.

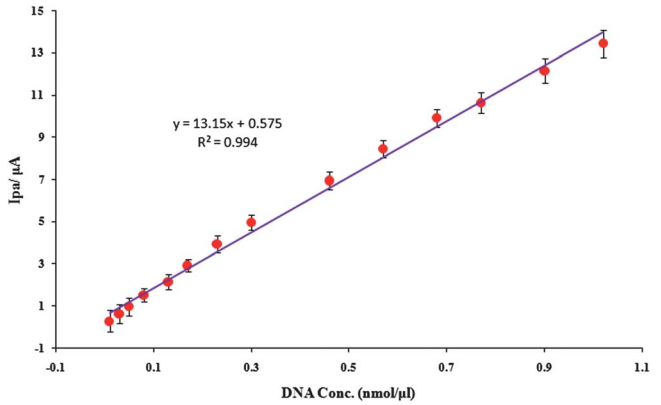


Fig. 7 Dependence of the observed peak current against ssDNA concentrations based on guanine oxidation signal at the CD/PNAANI/CNT modified SPE in 0.01 M PBS (pH 7.4).

Under the optimum conditions the linear dependences of the **G** and **A** oxidation peak current with the ssDNA concentration was studied by CV (Fig. 6) and DPV. A linear regression equation was obtained in the concentration range from 0.01 to 1.02 nmol μL^{-1} with a detection limit of down to 5 pmol μL^{-1} (Fig. 7).

The relative standard deviation (RSD) for four independent measurements of fixed concentration of ssDNA was 4.8%. So the proposed method could be successfully applied for the detection and quantification of ssDNA in its low concentration level.

4. Conclusion

We described a novel, simple and reliable method for amplifying label-free electrochemical detection of DNA hybridization based on the enhanced voltammetric signals of the **G** and **A** nucleobases at a CD/PNAANI/CNT modified screen printed electrode. CD and CNT as modifier, not only significantly improve the oxidation currents of **G** and **A** residues, but also negatively shift their oxidation potentials and facilitate the detection of DNA and DNA hybridization events. The detection scheme is also able to discriminate between targets (complementary), noncomplementary, and two-base mismatch sequences. The proposed method, is a simple, rapid, inexpensive, and sensitive electrochemical method for the determination of DNA without any external labels such as intercalators, metal complexes, organic dyes or thiol/amino-labels. The ssDNA fragment could be selectively detected by the proposed electrochemical DNA biosensor with a detection limit of 5 pmol μl^{-1} and a linear range of 0.01 to 1.02 nmol μl^{-1}.

Acknowledgements

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