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Short communication

Electrochemical detection of enzyme labeled DNA based on disposable pencil graphite electrode

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Abstract

Electrochemical biosensor for the detection of DNA hybridization using the reduction signal of α-naphthol is described. A pencil graphite electrode was used as a working electrode. Capture probes were covalently attached on to the pencil graphite electrode surface (PGE) at the 5′ end amino group by using *N*-(dimethylamino)propyl-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (NHS) as a coupling agent on to PGE. After capture probe immobilization on to PGE surface; probe was hybridized with complementary biotinylated oligonucleotide. Alkaline phosphatase labeled with extravidin (Ex-AP) binds to biotinylated hybrid via biotin–avidin interaction. α-Naphthyl phosphate (α-NAP) was added and the reaction between alkaline phosphatase (AP) and α-NAP was occurred consequently as a substrate of AP, α-NAP reduction signal was obtained from this reaction, at −0.100 V by using differential pulse voltammetry (DPV). Other experimental parameters were studied such as; optimizations of hybridization time, and the concentrations of capture probe, biotinylated oligonucleotide and enzyme.

Keywords: DNA biosensors; α-Naphthol; Alkaline phosphatase; Electrochemistry; Pencil graphite electrode

1. Introduction

Recently in diagnostic studies, DNA analysis is steadily gaining importance. DNA biosensor technologies are currently under intense investigation for their rapid, low cost, and reliable detection of specific DNA sequences in human and microbiological nucleic acids [1–3]. Recently many electrochemical methods detecting DNA hybridization have been reported [4,5]. One of these electrochemical methods is based on enzyme–substrate labeling that is the alternative approach to hybridization detection [6,7]. These include reduction of H_2O_2 signal, binding the nucleic acids to the surface of the electrode with biotin–avidin, digoxigenin–antidigoxigenin conjugation [8,9].

The biotin–avidin system is used in a variety of biotechnological and diagnostic applications. It involves a chemical [10] or genetic (the bio-tag biotinylation) biotinylation step [11].

Extravidin is a tetrameric protein like streptavidin which binds very tightly to the small molecule, biotin. The binding constant for this interaction is very high and has made the extravidin–biotin system the focus of a number of studies aimed at determining what particular intermolecular interactions give rise to the tight binding [12,13].

Alkaline phosphatase (AP) is a periplasmic protein in *E. coli*, which is dimeric with a molecular weight of 94,000. If retained in the cytoplasm, AP is enzymatically inactive [14–16]. Many enzyme immunoassays use alkaline phosphatase as a label. Recently, electrochemical detection methods have gained attention as it is possible to measure the current precisely even in coloured and turbid samples.

It was reported here as the first time that the hybridization detection of enzyme labeled DNA via covalently immobilization technique was developed by using α-NAP signal; an electroactive reporter. AP has been immobilized at an electrode surface via biotin–extravidin linkage and

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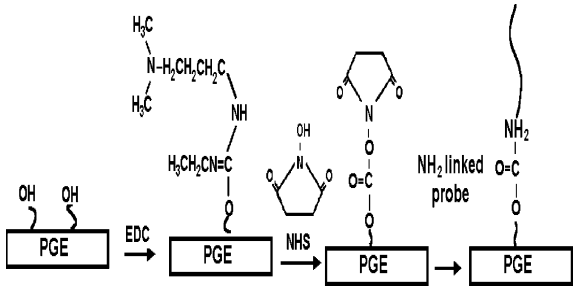


Scheme 1. Schematic presentation of probe immobilization onto pencil graphite electrode surface.

assayed electrochemically by using α-NAP as a substrate for the hybridization detection. The capture probes were covalently attached onto the PGE surface at the 5′ amino groups by using EDC and NHS as coupling agents (Scheme 1). The attached probes on PGE surface were hybridized with biotinylated oligonucleotides by immersing the electrodes in target solution. Alkaline phosphatase labeled extravidin (Ex-AP) bind to hybrid, α-naphthyl phosphate (α-NAP) was added and the reaction between alkaline phosphatase (AP) and α-NAP was occurred consequently as a substrate of AP. After hybridization occurred onto PGE surface the reduction signal of α-naphthol was measured by differential pulse voltammetry (DPV).

2. Experimental

2.1. Apparatus

The reduction signal of α-naphthol was investigated by using DPV with an AUTOLAB PGSTAT 30 electrochemical analysis system and GPES 4.8 software package (Eco Chemie, The Netherlands). The three-electrode system consisted of the pencil graphite electrode (PGE) as the working electrode, the reference electrode (Ag/AgCl) and a platinum wire as the auxiliary electrode. A Noki pencil Model 2000 (Japan) was used as a holder for the graphite lead (Pentel or Tombo, Japan). Electrical contact with the lead was obtained by soldering a metallic wire to the metallic part.

2.2. Materials and reagents

All stock solutions were prepared using ultra-pure deionized water. *N*′-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) was obtained from Aldrich (Germany), *N*-hydroxysuccinimide (NHS), A-conjugated extravidin. α-Naphthyl phosphate was obtained from Sigma (Germany). Other chemicals were of analytical reagent grade:

- capture probe (24 base sequence): NH_2-5′-CCT GCC CCA ATC CCT TTA TTA CCC-3′;
- complementary biotinylated oligonucleotide (25 base sequence): bio-5′-G GGG TAA TAA AGG GAT TGG GGC AGG-3′;
- noncomplementary biotinylated oligonucleotide (24 base sequence): bio-5′-TTT CAA AAT ATC GCG TAA GTC TCC-3′.

The oligonucleotide stock solution (100 mg/L) was prepared with TE solution (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and kept frozen.

The in-house distilled and deionized water was used in all solutions.

2.3. Procedure

Each measurement involved the immobilization/detection cycle at a fresh PGE surface. All the experiments were performed at room temperature (25.0 ± 0.5) °C.

2.4. Probe immobilization onto the electrode surface

The amino-linked capture probe was immobilized on to the electrode surface by covalent activation by immersion in the 50 mM phosphate buffer (pH 7.4) containing the covalent agents 5 mM EDC and 8 mM NHS for 1 h [2,5]. After activation of the electrode surface with the coupling agents, electrodes were immersed into the phosphate buffer (pH 7.4) containing 10 ppm capture probe for 1 h, the capture probe was immobilized onto PGE surface from amino-link at the 5′-end (Scheme 2). Thus a probe modified PGE was obtained.

2.5. Hybridization at the electrode surface

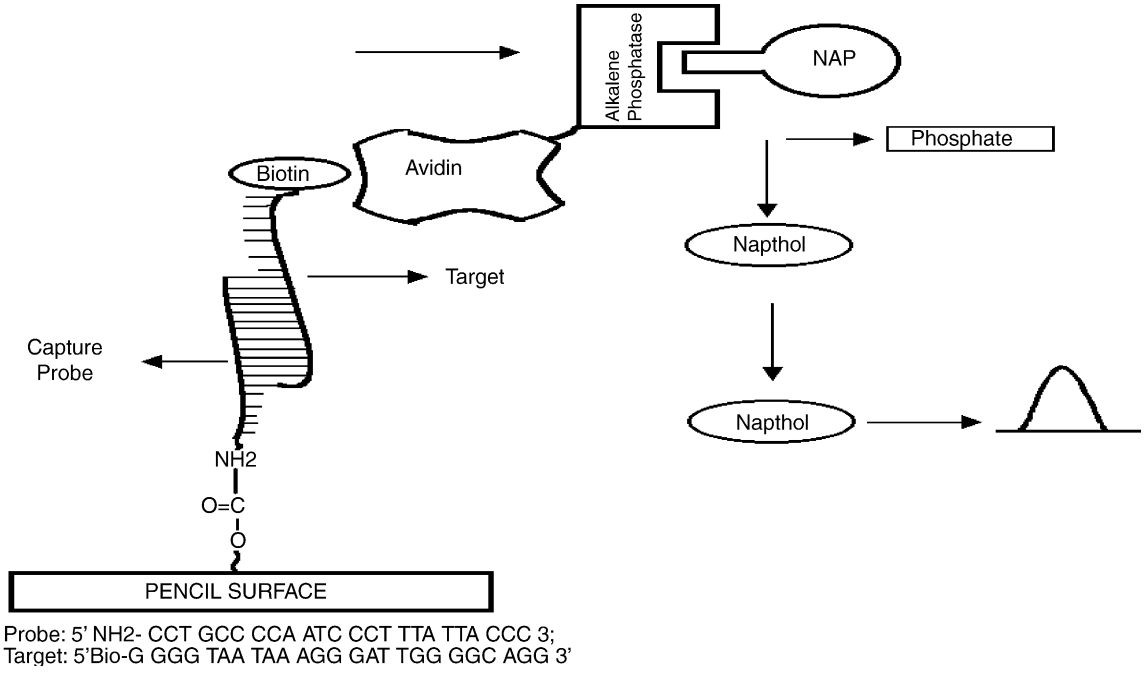
The capture probe modified PGE was immersed into the phosphate buffer (pH 7.4) containing complementary oligonucleotide of which was labeled with biotin at the 5′ end for 1 h. And the electrode was rinsed with PBS to remove the nonspecific adsorption. Hybridization between capture probe and noncomplementary oligonucleotide was monitored following the same method as described above.

2.6. AP labeled extravidin binding to the hybrid

After hybridization between capture probe and biotinylated oligonucleotide on to PGE surface, electrodes were immersed in to the phosphate buffer (pH 7.4) containing 150 μg/mL alkaline phosphatase labeled extravidin conjugate for 1 h. The affinity between extravidin and biotin is too strong and specific that they bound each other strongly.

2.7. Reaction between α-naphthyl phosphate and alkaline phosphatase

α-Naphthol occurred at the electrode surface by the reaction between alkaline phosphatase and α-naphthyl phosphate by immersing the electrode, which was coated with Ex-AP labeled hybrid on its surface, into the Tris–HCl (pH 9.6) containing 10 mM α-naphthyl phosphate for 1 h.



Scheme 2. Schematic presentation of DNA hybridization and detection procedure.

2.8. Voltammetric transduction

The reduction signal of the α-naphthol occurred at the electrode surface was measured at −0.1 V by using differential pulse voltammetry (DPV) in the 50 mM phosphate buffer (pH 7.4). DPV was performed by scanning scanning between +0.4 and −0.2 V at 50 mV/s pulse amplitude and 16 mV/s scan rate. Repetitive measurements were carried out by repeating the above assay format.

3. Results and discussion

The electrochemical response of α-naphthol that was occurred in pretreated and unpretreated PGE surface was also investigated and it was observed that α-naphthol reduction signal obtained from pretreated PGE surface is higher than the signal obtained from unpretreated PGE surface. But there is no difference between responses obtained from complementary and noncomplementary targets for each pretreated and unpretreated PGE surface. Thus the procedure was performed without pretreatment step of graphite electrode.

The interaction of AP with α-naphthyl phosphate (α-NAP) resulted in a formation of α-naphthol, which is measured voltammetrically with pencil graphite electrode and has been applied to AP-labeled electrochemical detection assay. The oxidized α-naphthol signal is at −0.100 V.

The DPV reduction signals of α-naphthol at PGE was measured in each conditions, they are shown in Fig. 1. The signal of α-naphthol obtained from hybrid is higher than the ones obtained from noncomplementary probe, blank containing EX-AP, and only α-naphthyl phosphate. This is due to the affinity between avidin and biotin, after hybridization with probe biotinylated target. AP labeled extravidin (Ex-AP) bounds to this hybrid and after accumulation of α-naphthyl phosphate, AP reacts with α-naphthyl phosphate and consequently α-naphthol occurs.

The electrochemical response of α-naphthol at PGE surface was also investigated (not shown). It was observed that α-naphthol had an electrochemical response in the potential range scanned in this study. And the response was two times higher than the α-naphthol occurred after the reaction between α-naphthyl phosphate and AP.

Fig. 2 shows the effect of probe concentration hybridized with the same biotinylated oligonucleotide concentration. It was observed that; α-naphthol reduction signal increased

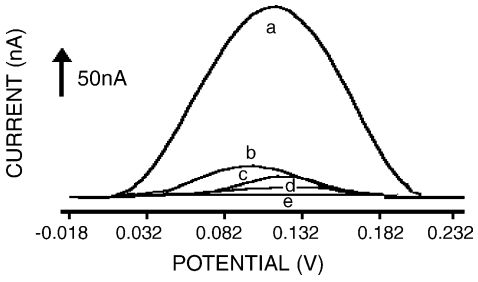


Fig. 1. Differential pulse voltammograms of α-naphthol reduction signal, obtained after the reaction between alkaline phosphatase and α-naphthyl phosphate; before probe immobilization (a) bare, α-naphthyl phosphate; (b) blank, containing only Ex-AP; (c) after probe immobilization. Hybridization signal with (d) complementary biotinylated oligonucleotide; (e) noncomplementary biotinylated oligonucleotide. DPV measurement, in a blank 50 mM phosphate buffer (pH 7.4) containing 20 mM NaCl as scanning between +0.4 and −0.2 V at 50 mV/s pulse amplitude and 16 mV/s scan rate.

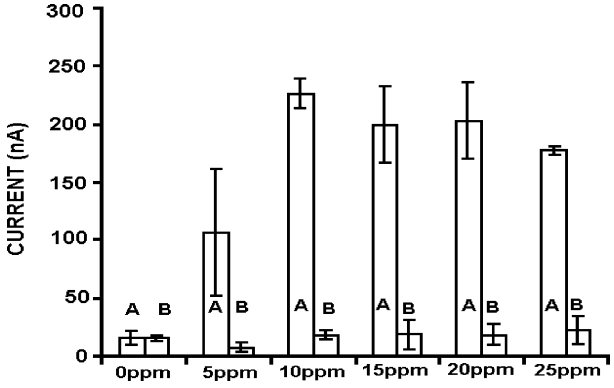


Fig. 2. Histograms based on α-naphthol reduction signal obtained from various probe concentrations after hybridization with in 15 μg/mL concentration of: (A) complementary biotinylated oligonucleotide; (B) noncomplementary biotinylated oligonucleotide. Other conditions are as in Fig. 1.

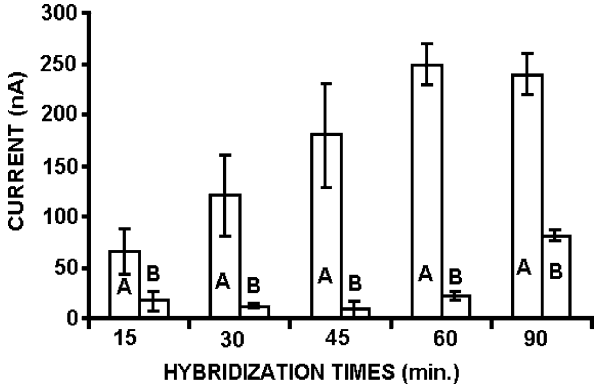


Fig. 4. Histograms based on α-naphthol reduction signal obtained after hybridization with: (A) complementary biotinylated oligonucleotide; (B) noncomplementary biotinylated oligonucleotide, in various hybridization times. Other conditions are as in Fig. 1.

gradually with concentration up to 10 μg/mL capture probe above, which it stared to level off till 25 μg/mL. The optimum probe concentration was chosen as 10 μg/mL.

The effect of biotinylated complementary oligonucleotide concentration with the same amount of probe was presented in Fig. 3. It was observed that; the response of α-naphthol decreased when the biotinylated oligonucleotide was also decreased till 15 μg/mL. The optimum biotinylated oligonucleotide concentration was chosen as 15 μg/mL.

The effect of hybridization time was also studied in Fig. 4. It was observed that response of α-naphthol obtained from 60 min hybridization time is higher than the responses obtained from 15, 30 and 45 min. The 60 min was chosen as the optimum hybridization time.

The effect of enzyme amount of the response α-naphthol signal was studied in various concentrations during hybridization step, such as: 600, 300, 150, 75 and 37.5 μg/mL concentrations of AP in Fig. 5. It was observed that the signal of α-naphthol was increased gradually till 150 μg/mL concentration of Ex-AP above, and then it stared to level off till 37.5 μg/mL. The optimum Ex-AP concentration was chosen as 150 μg/mL.

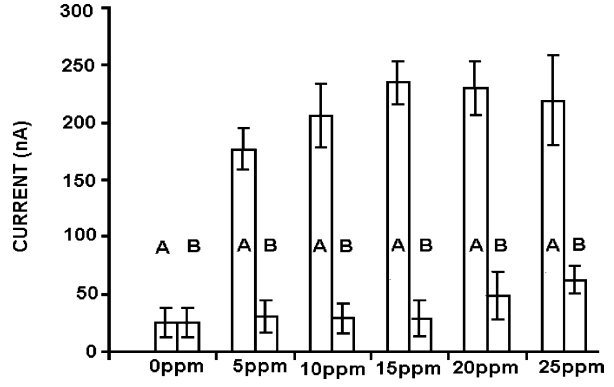


Fig. 3. Histograms based on α-naphthol reduction signal obtained after hybridization with 10 μg/mL capture probe concentration and various biotinylated oligonucleotide concentrations: (A) complementary biotinylated oligonucleotide; (B) noncomplementary biotinylated oligonucleotide. Other conditions are as in Fig. 1.

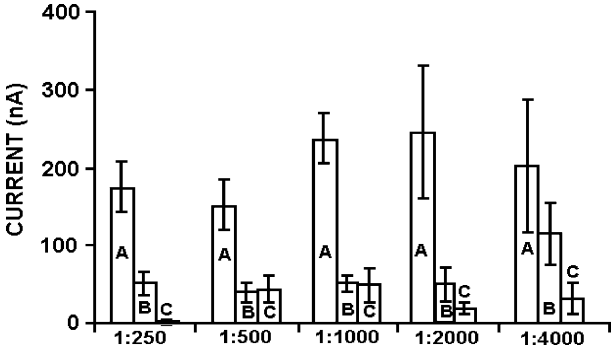


Fig. 5. Histograms based on α-naphthol reduction signal obtained after hybridization with: (A) complementary biotinylated oligonucleotide; (B) noncomplementary biotinylated oligonucleotide; (C) before hybridization in various enzyme amounts. Other conditions are as in Fig. 1.

4. Conclusion

Electrochemical detection of biological compounds in coupling with enzymes was performed greater selectivity and sensitivity [17,18].

The electrochemical DNA biosensor represented in this study offers fast and reliable results for detection of DNA hybridization is described. The immobilization method reported here, has been very useful for monitoring the DNA hybridization on the PGE surface. α-Naphthol is an electroactive reporter, which occurs after the reaction between biotinylated hybrid and Ex-AP.

Coming applications by using this effective sensor with α-naphthol signal as an electroactive reporter occurring after reaction between biotinylated hybrid and Ex-AP for the detection of microbiological and inherited diseases are being explored in this laboratory.

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