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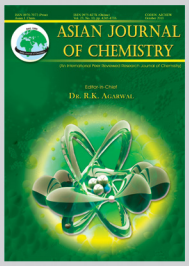

Electrochemical DNA Biosensor Based on Poly(allylamine hydrochloride) Modified Screen Printed Electrode

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An electrochemical method was used to characterize the interaction between Hoechst 33258 and the short DNA sequence related to *E. coli lac* gene. The probe ssDNA was immobilized on poly(allylamine hydrochloride) *via* physical adsorption and covalent attachment. The voltammetric signals of Hoechst 33258 have been investigated using screen printed electrode. The interaction of Hoechst 33258 with ssDNA and dsDNA has been studied and proven to have a promising strategy in improving the selectivity of the DNA sensor. The extent of hybridization was evaluated on the basis of the difference between signals of Hoechst 33258 with a probe DNA before and after hybridization with the target ssDNA. Control experiments with non-complementary were performed to test the selectivity of the developed DNA biosensor. The redox current was linearly related to the concentration of the target ssDNA from 1.5-6 μM with a detection limit of 0.6 μM.

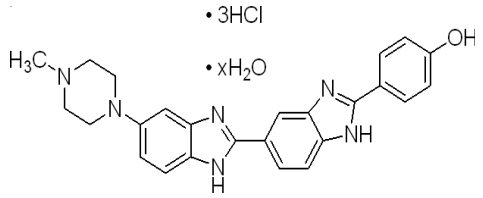
Key Words: Hoechst 33258, DNA biosensor, Hybridization, Poly(allylamine hydrochloride), Screen printed electrode.

INTRODUCTION

The detection of DNA hybridization is of central important to the diagnosis and treatment of genetic diseases, for the detection of infectious agents and for reliable forensic analysis. A variety of techniques have been developed for the detection of DNA hybridization, such as electrochemical, fluorescent, radiochemical, piezoelectronic, surface plasmon resonance spectroscopy and quartz crystal microbalance. Such techniques rely on conversion of the DNA base-pair recognition event into a useful detection signal. Among them, electrochemical technique is very attractive owing to rapid and direct detection of DNA hybridization, high sensitivity, inherent simplicity and miniaturization.

DNA biosensors consist of a single stranded DNA probe coupled to an electrochemical transducer that converts a recognition event between complementary DNA sequences into a measurable voltammetric, chronoamperometric or impedimetric signal. In the conventional electrochemical method for DNA detection, DNA probes are immobilized on the surface of an electrode and target DNA is hybridized to the probes on the surface.

A variety of approaches have been explored in electrochemical detection of DNA hybridization, mainly based on the current signal generation by direct oxidation of guanine base[1], by double strand DNA intercalators such as cationic metal complexes; ruthenium bipyridine[2], cobalt phenanthroline[3], anticancer drug *i.e.*, daunomycin[4], epirubucin[5], echinomycin[6], organic dye[7] and metal nanoparticles labels[8]. Among these hybridization indicators, DNA intercalators has received much attention due to its simplicity compared to using redox label and its sensitivity compared to direct oxidation of guanine base. The interaction of DNA with small molecules can result in the formation of DNA-molecule biocomplexes and have a large influence on the properties of DNA. Recently, the interaction of toxic chemicals and drugs with DNA was widely studied by an electrochemical method. Wang *et al.*[9] established an electrochemical equation for examining the interaction of irreversible redox compounds (Hoechst 33258) with DNA. The electrochemical behaviour of Hoechst 33258 and its interaction with DNA was investigated using cyclic voltammetry and other electrochemical techniques. The results showed that Hoechst 33258 bind tightly to the minor groove of DNA and cover four base pairs. Hoechst 33258, 2-(4-hydroxyphenyl)-5-[5-(4-methylpiperazine-1-yl)benzimidazo-2-yl]-benzimidazole molecular structure shown in **Scheme-I**), a fluorescence histochemical stain is an *N*-methyl piperazine derivative with two benzimidazole groups and one phenol group and is the best known of the *bis*-benzimidazole family of minor groove binders. It is a long, flexible molecule with a positively charged end and a number of proton donor and acceptor groups for possible hydrogen bonds spaced between[10].



Scheme-I : Molecular structure of Hoechst 33258

The immobilization of DNA onto the electrodes plays an important role in the overall performance of the DNA electrochemical detection. Several methods for immobilization DNA probes onto electrode surfaces have been reported. These include chemisorption[11], physical adsorption[12], entrapment in a gel or polymer[13], covalent binding[14], cross linking and electrochemical polymerization[15].

Using a polymeric support on a conducting surface is a convenient way of increasing the hybridization signal by deposition of more probe oligonucleotides due to the higher active area. The polymeric support provides a suitable interface for grafting DNA probes onto electrode surfaces due to numerous attractive properties including strong adsorptive capabilities, good ion-exchange capacity and fine conductivity at room temperature for conducting polymer. Attachment of oligonucleotides to the polymer support may be done in a variety of ways, before, during or after deposition of the polymer matrix[16].

The aim of this work is to investigate an alternative approach through immobilization of DNA probe on polyelectrolyte poly(allylamine hydrochloride) for the electrochemical detection of DNA hybridization. In present work an electrochemical method for the detection of DNA hybridization employing Hoechst 33258 as an electrochemical intercalator and utilizing polyallylhy-drochloride for the immobilization of DNA probe was studied. When the hybridization reaction of the immobilized ssDNA probe with the target ssDNA occurs and forms the dsDNA duplex, Hoechst 33258 intercalates into the dsDNA duplex and gives a higher current response than that of ssDNA. In this write up, immobilization procedure, optimization of the assay condition and analytical performance are presented.

EXPERIMENTAL

Poly(allylamine hydrochloride) (Aldrich) and Hoechst 33258 (Sigma) were kept refrigerated prior to use. Stock solutions of Hoechst 33258 (1 mM) were prepared in a 0.1 M KCl (pH 7). Poly(allylamine hydrochloride) solutions (17.9 mM) were prepared in deionized water. The tested oligomers were synthesized by Sigma Genosys. Their base sequences were as follows: a) 25-mer probe (ECIPROBE): 5' CAG GAT ATG TGG CGG ATG AGC GGC A -3'; b) 25-mer target DNA (ECICOMP): 5' TGC CGC TCA TCC GCC ACA TAT CCT G-3'; c) 25-mer non-complementary (ECINCOMP): 5'GGC CAT CGT TGA AGA TGC CTC TGC C-3'.

DNA oligonucleotide stock solutions (1.3 mM) were prepared in a phosphate buffer (pH 7) and stored in -20 ºC. All other reagents were of analytical grade and Millipore Milli-Q ultrapure water was used throughout.

Electrochemical measurements were performed with a Solartron 1286 potentiostat. A three channel screen printed electrode (spe) composed of carbon paste electrode as the working electrode, a carbon paste counter electrode and Ag/AgCl as the reference electrode, were used.

Hybridization: The ssDNA modified electrode was immersed in hybridization buffer (0.05 M phosphate buffer with 0.75 M NaCl at pH 7) containing target ssDNA for 40 min at 38 ºC with mild stirring to form dsDNA at the electrode surface. After hybridization, the dsDNA electrode was washed with 0.02 M Tris HCl (pH 7) containing 20 mM NaCl and water to remove non-specific bound DNA. The electrode was then immersed in 1 mM Hoechst 3258 for 10 min under dark condition and washed with phosphate buffer. Cyclic voltammograms experiments were carried out by immersing the desired electrode in 0.2 M phosphate buffer solution. The anodic peak current derived from the Hoechst 33258, associated with the hybrids formed on the electrode was used to quantify the target DNA.

RESULTS AND DISCUSSION

Immobilization of ssDNA probe: Techniques commonly used for immobilization of DNA onto solid substrates are layer-by-layer (LbL) self assembly by electrostatic interaction and Langmuir-Blodgett (LB) technique[12]. Layer-by-layer films are predominantly assembled by the alternate deposition of positively and negatively charged polymers facilitated by electrostatic interaction.

Utilizing this principle the present investigation has been carried out to study the immobilization of ssDNA by layer-by-layer technique through electrostatic interaction of polyanionic ssDNA with a polycationic poly(allylamine hydrochloride). Poly(allylamine hydrochloride) is a polyelectrolyte with a basic nature (pKa = 9.67), equivalent monomer with both hydrophilic parts (amino groups and their counter ions) and hydrophobic parts (hydrocarbon backbone). Poly(allylamine hydrochloride) has been used for the preparation of multilayer from analytical sensors to nanofiltration membranes or controlled release materials. The common procedure for electrode coating with poly(allylamine hydrochloride) is based on solvent evaporation. In pure water solution poly(allylamine hydrochloride) assumes a linear conformation where the polymer chain is expanded producing very thin assembled film[17].

Polymer coatings on the electrode were obtained by solvent evaporation by application of a microdroplet of the polymeric solution with an appropriate polyallylaminehydrochlo-ride concentration directly on the surface of the working electrode (on screen printed electrode). The electrode was then exposed to the 0.3 mM ssDNA solution. Multilayer films were then constructed by continuing this sequential adsorption process, alternating between positive [poly(allylamine hydrochloride)] and negative (ssDNA) polyion solutions until the desired layer number was reached. After each deposition, the films were dried under a stream of high purity nitrogen.

Covalent immobilization appears to be one of the most appropriate techniques for fabrication of DNA hybridization electrodes. This is because the probe DNA is held onto transducer surface at one end having backbone of the single stranded DNA free onto the surface giving desired flexibility and pace to hybridize with the complementary strand.

N-(3-dimethylaminopropyl)-*N*-ethyl-carbodiimide hydrochloride (EDC) and *N*-hydroxy-succinimide (NHS) were used to activate 5'- phosphate group of probe ssDNA suspended in Tris-EDTA buffer and the activation was done for about 18 h at 8 ºC. The activated probe ssDNA was then immobilized onto the poly(allylamine hydrochloride) layer deposited on the surface of the electrode.

Interaction of ssDNA and dsDNA with Hoechst 33258 in bulk solution: Fig. 1 shows the cyclic voltammograms (CV) of bare screen printed electrode (spe) in 0.2 M PBS containing 0.001 M Hoechst 33258 and also cyclic voltammograms for mixture of Hoechst 33258 with ssDNA and dsDNA. Hoechst was irreversibly oxidized at 0.4 V with high current density. In the presence of ssDNA, there are no significant changes on the peak compared to the original cyclic voltammograms of Hoechst solution. However a sharp pre-peak was observed before the actual anodic peak indicating adsorption of Hoechst on the surface of the electrode. Addition of dsDNA however, results in a shift in the peak potential to a more negative value and the current decreased sharply compared to without DNA. A great decrease in current can be attributed to a decrease in the rate of diffusion of Hoechst bound to dsDNA with large molecular weight. Scan rate study revealed that I_{pa} values were linearly dependant on square root of the scan rate which indicates an irreversible electrode process with diffusion controlled electrochemical process.

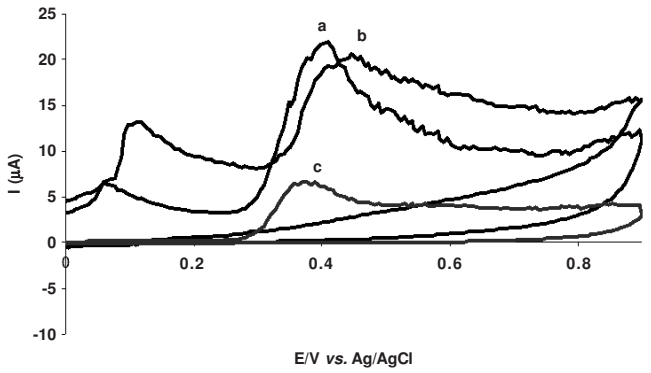


Fig. 1. Interaction of ssDNA and dsDNA with Hoechst 33258 in bulk solution (scan rate 50 mV/s)

Electron transfer between electroactive species and electrode occur *via* quantum mechanical tunnelling between the two locations. The electroactive species must be located 10-20 Å of the electrode surface to undergo electro reduction[16]. Electrochemical species must first diffuse from bulk solution within the critical distance for electron tunnelling before electron transfer takes place. The decrease in the rate of diffusion will reduce the rate of electron transfer which results in a decrease in redox current.

Interaction of ssDNA/spe and dsDNA/spe with Hoechst: Further investigation was carried out on the interaction of Hoechst with immobilized ssDNA and after hybridization with target DNA. Response towards non-complementary DNA was also investigated.

Figs. 2 and 3 showed the signal of ssDNA modified spe incubated with Hoechst, after hybridization with the same amount of complementary DNA sequence and the non-complementary sequence for different immobilization procedures. Significant increase in the anodic current was observed after hybridization with the complementary ssDNA. The increment in anodic current is due to Hoechst concentrated on the electrode surface as a result of hybridization. Experiments showed that I_{pa} of Hoechst-dsDNA complex was in relation with the scan rate. The I_{pa} values were linearly dependant on scan rate, which indicates an electrochemical process with surface adsorption. The peak to peak separation is more than 57 mV confirming an irreversible electrode process. The peak current almost did not increase when the DNA probe was exposed to the noncomplementary sequence (Figs. 2 and 3). This result indicated that only a complementary sequence could form a double strand DNA and cause a significant increase in the signal suggesting a high selectivity of the hybridization detection.

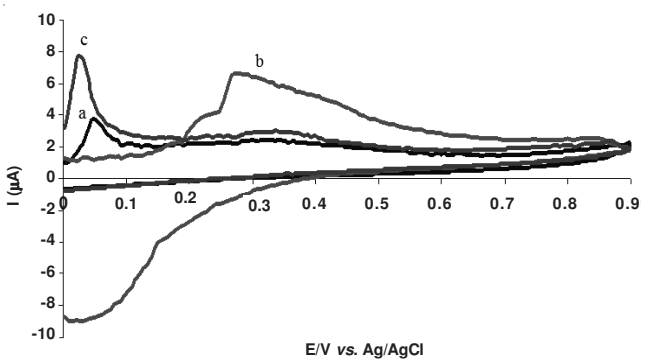


Fig. 2. Cyclic voltammograms of ssDNA modified spe incubated with Hoechst, after hybridization with the same amount of complementary DNA sequence and the non-complementary sequence utilizing direct physical adsorption on poly(allylamine hydrochloride) LBL (scan rate 50 mV/s)

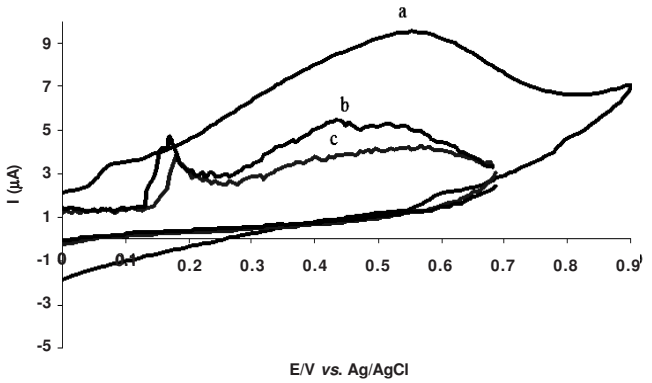


Fig. 3. Cyclic voltammograms of ssDNA modified spe incubated with Hoechst, after hybridization with the same amount of complementary DNA sequence and the non-complementary sequence utilizing covalent immobilization on poly(allylamine hydrochloride) LBL (scan rate 50 mV/s)

Optimization studies: Optimization studies were carried out to obtain reproducible signals with high sensitivity. The employed concentration of Hoechst (0.1-1 mM) was optimized by looking at the current produced. An increase in the response was observed up to 1 mM with a level off for higher concentrations. Accordingly, 1 mM Hoechst was used for further analytical procedure.

The reproducibility of the DNA sensors preparation was tested. Results for peak currents for 10 independently probe modified screen printed electrode (spe) constructed in the same manner yielded a R.S.D. of 7.2 and 9.5 % for covalent and layer-by-layer immobilization procedure respectively. These results demonstrated that the fabrication procedure of the DNA sensor was reliable, thus allowing reproducible electroanalytical responses to be obtained with different sensors.

Fig. 4 shows the influence of the hybridization time on the anodic current of Hoechst. It can be seen from the Fig. 4 that the oxidation current initially increases significantly with increasing hybridization time from 10 to 40 min and became almost constant after 40 min. This indicated that the hybridization reaction was dominantly completed after 40 min. Considering the sensitivity and assay time, therefore 40 min was chosen as the hybridization time.

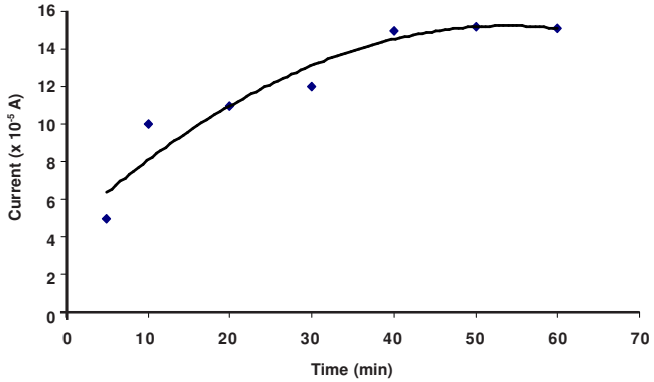


Fig. 4. Influence of the hybridization time on the anodic current of Hoechst 33258

Electrochemical response of a different concentration of complementary target: Under constant concentration of Hoechst, the response in the presence of hybridization between probe and increasing concentration levels of target was presented in Fig. 5. The cathodic and anodic current increased with increasing target concentration in the hybridization solution after the hybridization reaction (Figs. 2 and 3). The increase in anodic current *via* covalent attachment is more pronounced compared to physical adsorption hence it is used for analytical performance (Fig. 3). The increment is proportional to target concentration in the range of 1.5-6 µM [Fig. 5(a) and (b)] and the linear regression equation is I = 3.9 + 4.257 C (C is the concentration of target DNA, I is the anodic current) and the regression coefficient r = 0.9714. The limit of detection was calculated according to the 3a/m criteria, where m is the slope of the calibration plot and a is the standard deviation (n = 5). The calculated detection limit is 0.6 µM, which is an acceptable sensitivity taking into account that no signal amplification was required.

Selectivity is another major challenge in development of DNA biosensors. The detection of non-complementary DNA, mismatches and to differentiate the response of ssDNA and dsDNA have became a major criteria in development of DNA sensor. Most hybridization labels are cationic[18-21] in nature and the possibility of interaction between polyanionic DNA and the hybridization labels will limit the selectivity of the developed DNA sensor.

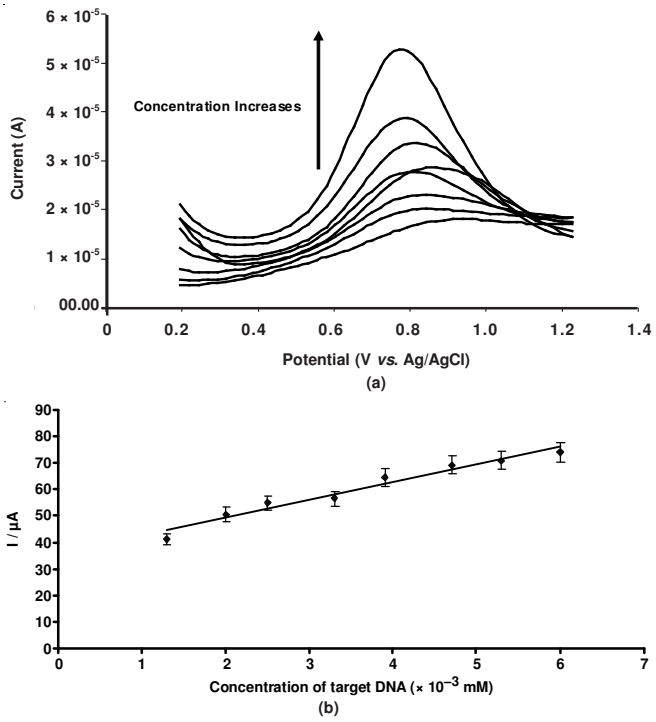


Fig. 5. (a) Differential pulse voltammogram (DPV) for different concentration of complementary target DNA (b) The calibration curve of the dependence of differential pulse voltammograms peak current on different concentration of target DNA. Amplitude: 0.05 V; pulse width: 0.06 s; pulse period: 0.2 s. Experimental condition as described in text

In this work, we have managed to offer sensitivity and selectivity through simple immobilization procedure and the use of minor groove binder as the hybridization label. The developed DNA sensor has been able to give a clear hybridization signal with acceptable selectivity towards target DNA. A satisfactory limit of detection has been achieved without the requirement of signal amplification.

Conclusion

A novel electrochemical detection of DNA hybridization based on physical and covalent attachment of probe DNA onto polyelectrolyte has been developed with high sensitivity and selectivity. The combination of screen printed electrode and the use of minor groove binder as the hybridization label has proven to have a promising strategy in improving the selectivity of the DNA sensor.

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