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Electrochemical probe DNA design in PCR amplicon sequence for the optimum detection of microbiological diseases

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Abstract

Direct electrochemical genosensor was developed for the detection of a probe sequence relative position in a PCR amplicon for the optimum detection of bacterial and microbiological diseases, in this study. The genosensor relies on a label-free electrochemical detection. The amino-linked inosine modified (guanine-free) coequal capture probes which were chosen from different parts of a PCR amplicon, immobilized on to disposable pencil graphite electrodes (PGE) by electrostatically and covalently. As a model case Hepatitis B virus (HBV) genome amplicon was used for the detection and specification. Hybridization was occurred after surface coverage with denatured amplicons. After hybridization, optimum probe sequence position was identified by using the differences between the responses of guanine oxidation signals. The results of this study might have a great convenience for the microbiological diseases detection applications such as DNA micro arrays.

Keywords: Electrochemical genosensor; DNA; PCR

1. Introduction

Specific DNA sequence detection provides the basis for monitoring a wide variety of infectious and inherited diseases. Molecular diagnostics based on the analysis of genomic sequences have offered a highly sensitive and quantitative method for the detection of infectious disease pathogens and genetic variations. Traditional DNA detection methods based on the coupling of electrophoresis separations and radio isotopic detection are labor intensive and time consuming, From this point of view, these methods are not well suited for routine and rapid medical analysis, particularly for point-of-care tasks. Recent advances in biosensors based on nucleic acids have led to the development of genosensor technology for sequence analysis [1–4].

Electrochemical hybridization biosensors hold a great promise in pathogen identification, mutation detection and genomic sequencing. They may greatly reduce the assay time and simplify the protocol [5–8]. Such fast on-site monitoring schemes are required for quick preventive action and early diagnosis. These novel sequence specific hybridization processes rely on monitoring the oxidation signal of most electro active bases of DNA [9–14], or with the signal of an electro active hybridization indicator which have different behaviors to single strand and double strand DNA [14–17]. The appearance of guanine signal after hybridization with inosine substitute probes and target sequences eliminate the external labels and shortened he assay time [18]. The oxidation signals of guanine have been employed to detect hybridization [19–20]. Such use of intrinsic signals of DNA is nowadays preferred because they shortened the hybridization detection assay time. In this report, the oxidation signals of guanine and adenine were also monitored for the hybridization detection.

Genosensors clarify the array technologies which are based on immobilized 15–25 mer single strand DNA called as capture probe molecules. These probe molecules are the complementary sequences of the specific part of the amplicon related to a virus, bacteria or a specific gene sequences [21–25]. The specificity and the selectivity of the hybridization detection by using long

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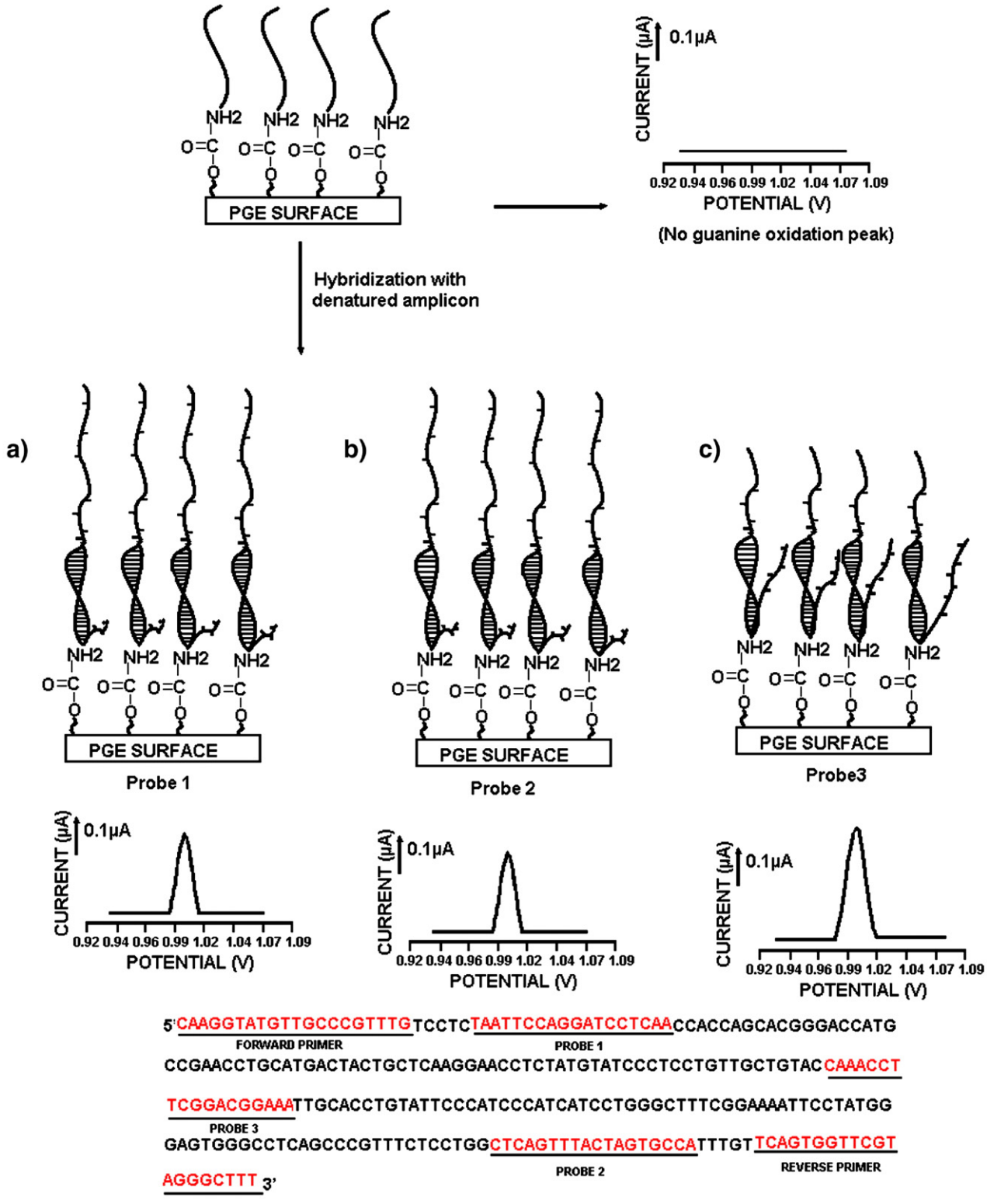

Fig. 1. Schematic presentation of study procedure.

amplicons are dependent on the conditions at the electrode surface such as; density, extent, and relative position of probe sequence.

Giallo et al. reported the steric factors of probe sequences in a long range amplicons. The authors have worked on enzyme-linked hybridization assay. Thiol-linked probe oligonucleotides that have been selected from several parts of a PCR amplicon. The detection was achieved after hybridization with three biotinylated signaling probes, to prevent the formation of duplex of the long amplicon [26]. Krull and co-workers worked

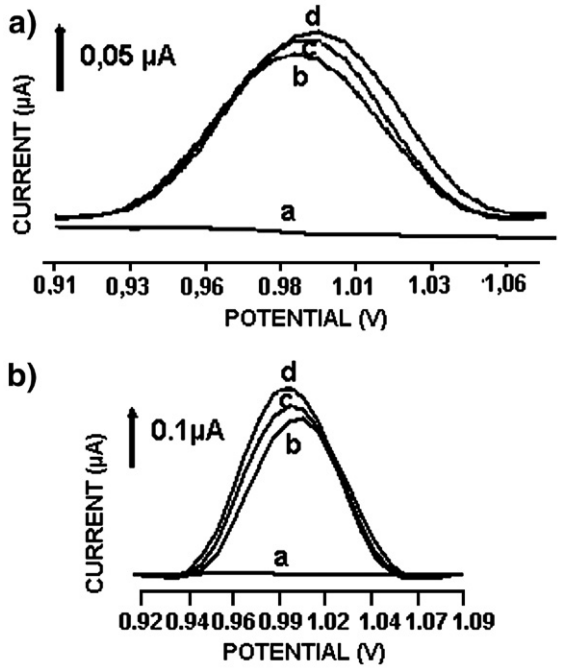

Fig. 2. Differential pulse voltammograms of guanine oxidation signals in acetate buffer solution obtained after probe immobilization by; a) electrochemically, b) covalently attachment; a) before hybridization, after hybridization between; b) hybrid (2Probe 2 with Target 2), c) hybrid 1 (Probe 1 and Target 1), d) hybrid 3 (Probe 3 and Target 3).

on the gradient of immobilized probe density, to clarify the effect of location and the extent of short oligonucleotide probes at selectivity for detection [27].

This report describes the effect of probe sequence configuration in a long amplicon for the selectivity of microbiological diseases detection. The study relies on hybridization detection via guanine oxidation peak by using three coequal probe oligonucleotides which were chosen from several parts of Hepatitis B virus (HBV) amplicon.

2. Materials and methods

2.1. Apparatus

Differential pulse voltammetry (DPV) was performed with an electrochemical analyzer Autolab PGSTAT 30 (Eco Chemie, The Netherlands) connected to a personal computer. Electrochemical measurements were carried out in a 10 mL voltammetric cell at room temperature (25 °C). The electrode system consisted of a platinum electrode that served as an auxiliary electrode, an Ag/AgCl as reference electrode and a disposable graphite electrode as working electrode (pencil graphite electrode — PGE) . A Noki pencil model 2000 (Japan) was used as a holder for graphite leads (Tombo, Japan). Electrical contact with the lead was obtained by soldering a metallic wire to the metallic part.

2.2. Reagents

N-hydroxysulphosuccinimide (NHS) and N-(3-dimethylamino) propyl)-N -ethylcarbodiimide hydrochlorides (EDC) were purchased from Sigma-Aldrich Chemical Company (Steinheim, Germany).

The PCR amplified real samples were kindly donated by the Department of Microbiology and Clinical Microbiology in the Faculty of Medicine, Ege University. The 18-mer synthetic oligonucleotides for the HBV (Inosine modified HBV probes and complementary targets) were purchased (as lyophilized powder) from TIB Molbiol Syntheselabor (Berlin, Germany); their base sequences are as below:

Probe 1 (18 mer-sequence complementary of 5 base distances from forward primer):

5′ NH_2 $(CH_2)_6$-TAA TTC CAI IAT CCT CAA 3′

Probe 2 (18 mer-sequence complementary of 5 base distances from reverse primer):

5′ NH_2 $(CH_2)_6$-CTC AIT TTA CTA ITI CCA 3′

Probe 3 (18 mer-sequence complementary of the inner part of amplicon):

5′ NH_2 $(CH_2)_6$-CAA ACC TTC IIA CII AAA 3′ (I = Inosine)

Probe 1, 2 and 3 are the complementary targets of different parts of the HBV amplicon. Probe 1 represents the 18 mer sequence, following 5 base after the forward primer, Probe 2 represents the 18 mer sequence 5 base prior to reverse primer, Probe 3 represents the 18 mer sequence at the middle section of the amplicon.

Each probe sequence contains inosine instead of guanine and 5 citosines to provide the response stability in comparison with each other.

Target 1 (18 mer-sequence, synthetic complementary of Probe 1): 5′ TTG AGG ATC CTG GAA TTA 3′

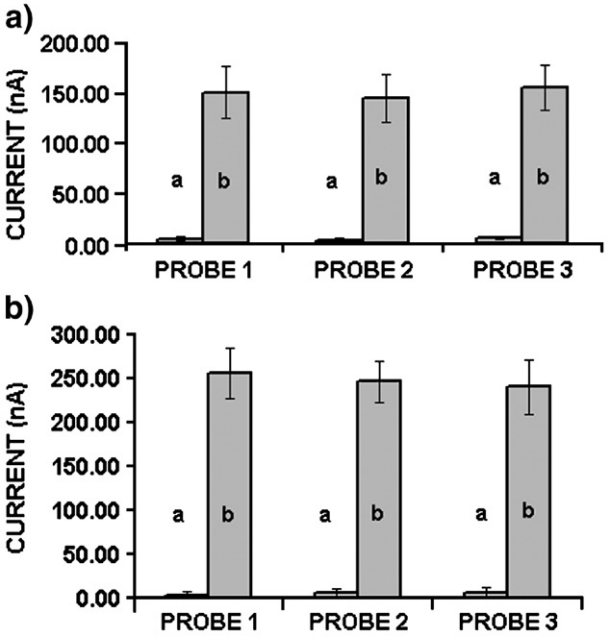

Fig. 3. Histammograms obtained from differential pulse voltammograms of guanine oxidation signals after probe immobilization by; a) electrostatically, b) covalently attachment; a) before hybridization, b) after hybridization with complementary targets.

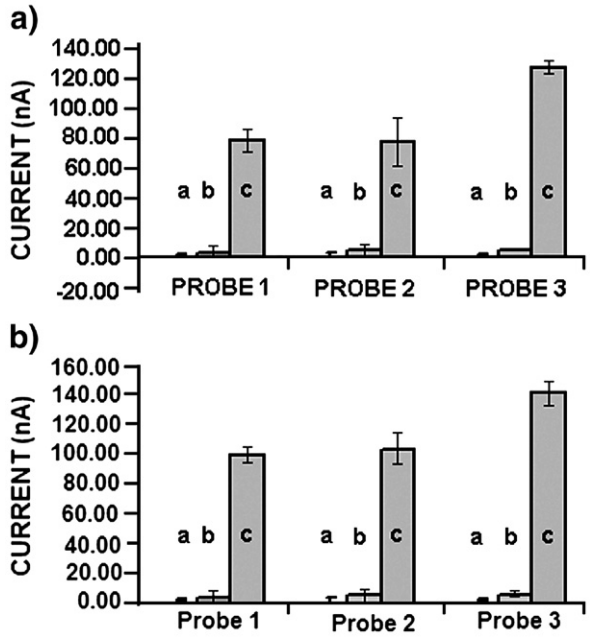

Fig. 4. Histammograms obtained from differential pulse voltammograms of guanine oxidation signals after probe immobilization by; a) electrostatically, b) covalently; a) before hybridization, after hybridization with; b) Non complementary PCR products (HSV real samples), c) HBV positive PCR products.

Target 2 (18 mer-sequence, synthetic complementary of Probe 2): 5′ TGG CAC TAG TAA ACT GAG 3′

Target 3 (18 mer- sequence, synthetic complementary of Probe 3): 5′ TTT CCG TCC GAA GGT TTG 3′

Non complementary Target

5′ CCG TCA GCT TGG TCA GGA C 3′

All oligonucleotide stock solutions (180 μmol/L) were prepared with ultra-pure distilled water and kept frozen. The results obtained from both techniques were compared within each other.

All stock solutions were prepared using deionized and autoclaved water.

2.3. Methods

2.3.1. PCR amplification of DNA from blood samples

DNA was extracted from venous blood by applying a salting out method. PCR amplification was performed in DNA Thermal Cycler (Perkin-Elmer Cetus) using oligonucleotide primers for HBV. Amplicon detection was done by electrophoresis of the end products in a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. The lengths of the markers used for the HBV electrophoresis analysis were 220 and 100 bp ladder (Roche Diagnostics, DNA molecular weight marker XIV), respectively. PCR working rules were strictly followed to avoid amplicon carry-over. All real samples were tested at least twice or three times by going back to a separate serum aliquot.

HBV PCR Product (263 bp sequence):

5′***CAAGGTATGTTGCCCGTTTG***TCCTC**TAATTCCAGGATCCTCAA**CCACCAGCACGGGACCATGCCGAACCTGCATGACTACTGCTCAAGGAACCTCTATGTATCCCTCCTGTTGCTGTAC**CAAACCTTCGGACGGAAA**TTGCACCTGTATTCCCATCCCATCATCCTGGGCTTTCGGAAAATTCCTATGGGAGTGGGCCTCAGCCCGTTTCTCCTG**GCTCAGTTTACTAGTGCCA**TTTGT***TCAGTGGTTCGTAGGGCTTT*** 3′

Non-complementary PCR Product (179 bp sequence):

5′ATC AAC TTC GAC TGG CCC TTC T TG CTG GCC AAG CTG ACG GAC ATT TAC AAG GTC CCC CTG GAC GGG TAC GGC CGC ATG AAC GGC CGG GGC GTG TTT CGC GTG TGG GAC ATA GGC CAG AGC CAC TTC CAG AAG CGC AGC AAG ATA AAG GTG AAC GGC AT***G GTG AGC ATC GAC ATG TAC GG*** 3′.

The bold and italic parts represent the forward and reverse primers, the bold and underlined parts represent the 3 probe sequences in PCR product sequence.

2.4. Hybridization detection

The hybridization detection was transduced by means of DPV with an AUTOLAB PGSTAT 30 electrochemical analysis system. The guanine oxidation peak height following base line fitting was used as the analytical signal.

The procedure of hybridization detection was performed by following steps; PGE preparation, electrode modification, probe immobilization, hybridization with synthetic oligonucleotides or denatured PCR amplicons, washing and voltammetric transduction on the surface.

2.4.1. Electrode (PGE) preparation

PGE's were Tombo leads with a diameter of 0.5 mm. The electrodes were immersed in to the measurement solution the working area was approximately 1.96 mm^2. The PGE was prepared by cutting the leads into 3 cm long sticks as described previously Ozkan et al and Kara et al. [18,20, and 22].

2.4.2. Electrode modification and probe immobilization

This procedure was established by using both electrostatically and covalently immobilization methods as follows;

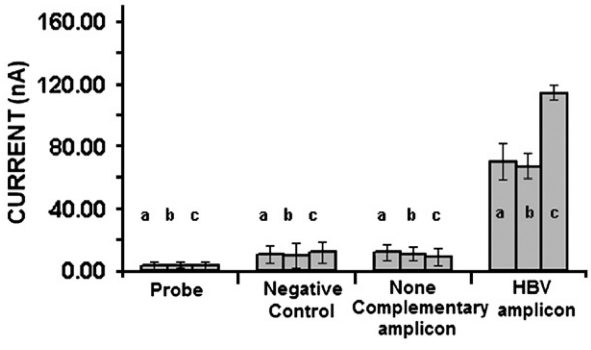

Fig. 5. Histammograms obtained from differential pulse voltammograms of guanine oxidation signals; a) Probe 1, b) Probe 2, c) Probe 3 modified PGE surfaces, before and after hybridization with positive HBV real samples, negative control samples, non complementary amplicon (HSV PCR products).

a) Elecrostatically immobilization method; The PGE's were activated by applying 1.40 V for 1 min in 0.05 mmol/L acetate buffer solution containing 20 mmol/L NaCl (pH 4.8) without stirring. The probe sequence was subsequently immobilized on to the pretreated PGE surface by applying a potential of 0.50 V at a concentration of 1.8 μmol/L containing acetate buffer solution (pH 4.80) at 200 rpm stirring, from negatively charged phosphate group. The electrode was then rinsed with acetate buffer solution (pH 4.80) with 20 mM NaCl for 10 s.

b) Chemically Immobilization Method: The PGE's were activated at 5 m mol/L EDC and 8 m mol/L NHS for 1 h. The probe sequence was subsequently immobilized on to PGE surface by covalently attachment from 5′ NH_2 $(CH_2)_6$ link.

2.5. Hybridization

After probe immobilization by electrostatically; the hybridization with synthetic targets (target 1, 2 and 3) and denatured PCR amplicon was performed by immersing the electrodes into the 2xSSC buffer containing target sequences for 6 min.

After covalently probe immobilization onto PGE surfaces hybridization was occurred with 2×SSC buffer containing synthetic targets and denatured amplicon sequences for 30 min. The electrodes were then washed with washing buffer as described above for 10 min.

Denaturation of PCR amplicons were occurred at 95 °C for 8 min and at 0 °C for 5 min [28]. The electrodes were then washed with 2×SSC+%0, 1 SDS as washing buffer for 1 min to prevent the unspecific bounding.

2.5.1. Voltammetric transduction

The electrodes were then transferred into the acetate buffer solution (pH 4.80) for voltammetric measurement. The differential pulse voltammograms were collected with amplitude of 10 mV at 20 mV/s scan rate. The raw data were treated using the moving average baseline correction with a "peak width" of 0.01 of GPES software. For the measurements performed with the bare PGE, no DNA was adsorbed onto the electrode surface. Repetitive measurements were carried out by renewing the surface and repeating the above assay format.

Control experiments were performed with real samples which contained HBV amplicons, negative controls, and non complementary (NC) targets.

The study procedure steps were illustrated in Fig. 1.

3. Results and discussion

In this work, a label-free electrochemical genosensor was developed for the study of microbiological disease detection selectivity. An electrochemical genosensor probe DNA sequence design for the sequence specific detection by using PGE as a disposable sensor devoted to microarray technology. The hybridization detection relies on differential pulse voltammetric transduction of the guanine oxidation after hybridization between; 1, 8 μmol/L probe 1 (following 5 base after the forward primer), probe 2 (5 base prior to reverse primer), probe 3 (at the middle section of the amplicon) and 2, 7 μmol/L target DNA sequences.

As mentioned above the inosine substituted (guanine-free) probe sequences thus no oxidation signal of guanine at about 1.00 V was observed before and after hybridization with negative control and NC sequences, as expected. The increase in the guanine oxidation signal shows that the target hybridized at the electrode surface. Therefore, no disparity in the guanine oxidation peaks was observed, between synthetic target sequences hybridized with probe oligonucleotides owing to their coequal base configurations [Figs. 2,3]. Thus the electrochemical detection protocols have been reported here; provides optimum sequence specific detection of the amplicon.

Fig. 2 shows the voltammograms of guanine oxidation signals obtained after electrostatically (Fig. 2A), covalently (Fig. 2B) probe immobilization and hybridization between 1,8 μmol /L inosine modified probe sequences (Probe 1, 2, 3) with 2, 7 μmol /L complementary target sequences (target 1, 2 and 3). No guanine signal was obtained before hybridization at probe modified PGE surface (a). It was not observed significant difference between the guanine signals obtained after hybridization with; b) probe 1 and target 1 (hybrid 1), c) probe 2 and target 2 (hybrid 2), d) probe 3 and target 3 (hybrid 3); owing to their coequal base configurations such as 18 mer inosine substitution and 5 citosines content.

The effect of experimental parameters including ionic strength, probe concentration, sample dilution, hybridization time were also achieved at optimum analytical performance [20,22,28]. Millan et al. reported that the higher association constant obtained at 20 mmol/L NaCl is expected, since the binding reaction is electrostatic in nature and electrostatic association is favored at low ionic strength [29].

Fig. 3 represents the histammograms with error bars of guanine oxidation signals obtained after hybridization between probe sequences with complementary targets by electrostatically (Fig. 3A) and covalently (Fig. 3B) probe immobilization technique. As expected, it was not observed any significant peak before hybridization and either significant differences between guanine peaks between hybrid 1 (a), hybrid 2 (b) and hybrid 3 (c).

Therefore; the differences between the guanine peaks obtained after hybridization between the HBV amplicons and each probe sequences accomplish the optimum probe design in an amplicon for the optimum microbiological disease detection.

Fig. 4 shows the histammograms based on guanine oxidation peaks obtained after hybridization with three coequal probes and HBV amplicon, to detect the effect of probe relative position in a long amplicon. Probe sequences were immobilized onto disposable PGE surface by electrostatically (Fig. 4A) and covalently (Fig. 4B) immobilization methods. Herpes simplex virus amplicon were used as non complementary target [22]. Interestingly the responses were obtained after hybridization with probe 3 which represents the 18 mer middle section of the amplicon; is significantly higher than the responses obtained after hybridization with probe 1 and probe 2 represents the 18 mer sequences at 5 base distant to forward and reverse primers.

Fig. 5 shows the histammograms that demonstrate the general evaluation of the optimum probe relative position in an amplicon for the hybridization detection. It was not observed an expressive guanine oxidation response from inosine substituted probe sequences modified PGE surfaces as expected, either the responses obtained after hybridization with negative control and non-complementary PCR amplicons. There is a significantly enhanced sensitivity was observed after hybridization with positive HBV amplicons. The differences between the responses obtained after hybridization with three coequal probes and positive amplicons shows the optimum probe DNA sequence relative position for optimum microbiological disease detection. Use of different positioned coequal base configured probe sequences specified the optimum microbiological disease detection. The highest performance was obtained after hybridization with probe sequence which takes a part at the middle section of the amplicon.

We hypotheses that; using probe sequences chosen from the beginning part of the amplicon; cause duplex formation at the posterior of the long sequence, in the contrast; the probe at the middle section prevent the duplex formation and stabilize the amplicon sequence for hybridization and provide an optimum diagnosis.

4. Conclusion

This study offered a new approach for the hybridization behavior and selectivity of three coequal probe oligonucleotides chosen from several parts of an amplicon related to a microbiological disease. An electrochemical probe DNA sequence design was investigated by using three guanine-free capture probes including coequal base configurations to provide an optimum detection for infectious diseases. The results obtained in this study have expressive practical consequences for sequence specific hybridization biosensor applications.

Aimed of this study, a label free electrochemical genosensor was designed for the optimization of the effect of probe sequence relative position in an amplicon related to a microbiological disease. Three 18 mer inosine substitute (guanine-free) capture probes including 5 citosines each were immobilized onto PGE surfaces by electrostatically and covalently attachment. Surface hybridization was monitored by using electrochemical guanine oxidation signal detection. As a model case HBV genome amplicon was used for the optimization and specification. The highest response obtained with the probe sequence which represents the middle section of the amplicon owing to its stabilization and prevention the formation of duplexes at long PCR product.

This procedure can be employed to detect specific gene sequences related to different viruses, bacteria or even inherited diseases for DNA microarray technology. Reports are in progress towards these directions.

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