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Coupling electrochemical response of a DNA biosensor with PCR for *Neisseria gonorrhoeae* detection ☆

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ABSTRACT

Early diagnosis of gonococcal infections is important with regard to a patient's health and stage of infection. In this context, we report the development of an *opa*-gene–based electrochemical DNA biosensor for detection of *Neisseria gonorrhoeae* by monitoring redox peak of methylene blue indicator. The fabricated biosensor has been shown to be highly sensitive and specific when evaluated with complementary, non-complementary, and 1-base mismatch DNA sequences and polymerase chain reaction (PCR) amplified products (amplicons) of standard strain of *N. gonorrhoeae* (ATCC49226). The biosensor has been further evaluated using amplicons of known positive and negative clinical samples, and cut-off for positives has been determined using receiver operating characteristic curve. The sensitivity (SN), specificity (SP), positive predictive value, and negative predictive value of the biosensor have been found to be 96.2%, 88.2%, 92.6%, and 93.8%, respectively. We conclude that the combination of PCR amplification with electrochemical detection shows distinct advantage of high SN and increased SP for gonococcal detection.

1. Introduction

In developing countries, sexually transmitted infections (STIs) and their complications are amongst the top 5 disease categories for which adults seek health care (UNAIDS, 1998). The currently available diagnostic testing algorithms require 2–14 days of turnaround time, thus contributing to low rates of patient return for test results, reinfection of the presenting patient, and ongoing transmission of infection in the patient's partner(s). Further, the stigma, privacy, and confidentiality issues associated with STIs emphasize the need for development of point-of-care (POC) or near POC diagnostics for these infections (Bissonnette and Bergeron, 2012; Hsieh et al., 2010). An ongoing revolution in this area is the development of biosensors that have the potential to be used as an integral component in POC test (POCT) devices.

DNA-biosensing methodologies are usually based on detection of DNA hybridization, which is both specific and robust (Drummond et al., 2003). However, very often, the amount of DNA to be detected (in femtomolar or attomolar ranges) is lower than the detection limit of conventional analytical techniques (Pedrero et al., 2011). One approach to overcome this problem would be amplification of signal using innovative routes of electrochemistry. However, it is pertinent to mention that amplification of the signal alone for detection of DNA, which may be present in a very low concentration in clinical samples, continues to be a challenge. Amplification of the target by means of polymerase chain reaction (PCR) before biosensor analysis is commonly required to overcome the problems imposed by the low abundance and extreme complexity of the non-amplified DNA in the clinical samples (Loaiza et al., 2009; Pedrero et al., 2011; Zhu et al., 2008).

Many attempts have been made worldwide for the development of electrochemical biosensors for pathogen detection (Gabig-Ciminska et al., 2004; Liao et al., 2006; Meric et al., 2002; Pedrero et al., 2011). However, most of these make use of synthetic DNA (complementary, non-complementary, and 1-base mismatch [OBM] sequences) for evaluation of the biosensor. Not much effort has yet been made on application of these methods to clinical samples. In a recent work

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(Singh et al., 2010), we reported fabrication of an electrochemical DNA biosensor based on self-assembled monolayer of thiolated DNA probe on gold electrode targeting *opa* gene of *N. gonorrhoeae*. Mercaptohexanol was used as a blocking agent, and methylene blue (MB), as the electro-active DNA hybridization indicator. The biosensor was used for successful detection of synthetic complementary DNA, PCR amplified DNA, and sonicated genomic DNA from gonococcal culture. In the present study, we have extended the approach to disposable screen printed gold electrodes for detection. Moreover, in this study, the performance of the biosensor has been validated using PCR amplified DNA (amplicons) from multiple known positive and negative clinical samples. During the study, it was observed that when the biosensor was evaluated on clinical samples, a quantitative variation in signal intensity was observed. This may perhaps be due to the variability in the bacterial load in clinical samples due to the host-pathogen interaction. In addition, a background current was detected, which was perhaps due to non-specific adsorption on the matrix. Keeping the above in mind, the present manuscript focuses on the study of the response characteristics of the fabricated bioelectrode with regard to the amplicons to arrive at a cut-off point using the receiver operating characteristic (ROC) curve to distinguish the infected from non-infected individuals (Akobeng, 2007). The diagnostic accuracy and the clinical utility index of the test have also been determined for validation of the assay.

2. Materials and methods

2.1. Primers and probe

Primers targeting *opa* gene of *N. gonorrhoeae* were used for PCR amplification, as described earlier (Verma et al., 2012) (GenBank accession no. PUID 9716120 SNUM 2706 Ng_opa). 5′-thiolated 19-mer oligonucleotide modified with a 6-carbon (C6) spacer arm was used as the probe (thssDNA). The sequences of oligonucleotide primers, probe, complementary, non-complementary, and OBM sequences used in this study are as follows:

Forward primer: 5′ CGGTGCTTCATCACCTTAG 3′
Reverse primer: 5′ GGATTCATTTTCGGCTCCTT 3′
Probe: 5′ S-S-C6-CGGTGCTTCATCACCTTAG 3′
Complementary sequence: 5′ CTAAGGTGATGAAGCACCG 3′
OBM: 5′ CTAAG**T**TGATGAAGCACCG 3′
Non-complementary sequence: 5′ ACGGTTCACGCGGTACTTA 3′

The primers were procured from Sigma-Aldrich (Co., St. Lious, MO, USA). The probe, complementary, non-complementary and OBM sequences were procured from Midland Certified Reagent Company (Midland, TX, USA).

2.2. Reagents

Screen-printed gold electrodes have been procured from DropSens (Oviedo, Spain). Tris buffer, ethylene diamine tetra acetic acid, potassium monohydrogen phosphate, potassium dihydrogen phosphate, and MB have been purchased from Sigma-Aldrich and 6-mercapto-1-hexanol (MCH) from Fluka (Gillingham, UK). All solutions and glassware have been autoclaved prior to being used. Solutions have been prepared in autoclaved deionized water (MilliQ 10TS, Millipore, Bedford, MA, USA).

2.3. Bacterial isolates and clinical samples

The bacterial strains used to test the specificity (SP) of the biosensor included *N. gonorrhoeae* (ATCC 49226), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Neisseria meningitidis* (ATCC 13077 serogroup A), and *Neisseria sicca* (ATCC 29193). The diagnostic stability of the probe sequence was further evaluated using 16 *N. gonorrhoeae* strains comprising of WHO B, WHO C, WHO F, WHO G, WHO K, WHO L, WHO M, WHO N, WHO O, WHO P, and 6 quality control strains derived from WHO Collaborating Centre for STD, The Prince of Wales Hospital, Sydney, Australia, and received from Regional Reference Laboratory, Safdarjung Hospital, New Delhi.

Patients in the age group of 15–45 years attending the STD Clinic, Dermatology OPD of AIIMS, New Delhi, India, with symptoms suggestive of gonorrhoea were included in the study. The inclusion criteria for patients were urethral, cervical/vaginal discharge, or lower abdominal pain. In addition, history of symptomatic partner or positive risk factors or partner with risk factors was taken into consideration. The swabs were collected in triplicate using standard techniques by a trained physician (WHO, 1999); one was used to prepare a smear for microscopic examination, second for culture, and third for DNA extraction for PCR followed by biosensor evaluation. This preliminary study with biosensor was performed on DNA extracted from 26 gonorrhoea-positive patient samples (i.e., positive by culture and PCR), 16 negative patient samples (i.e., negative by culture and PCR), and 1 negative control (without DNA). The study has been approved by the Ethics Committee, All India Institute of Medical Sciences, New Delhi, India.

2.4. Sample processing

Microscopic examination of smear was done following Gram's staining. Swabs were plated on standard culture media (Saponin lysed blood agar and chocolate agar), and identification was done as per the standard microbiological protocol (WHO 1999). DNA was extracted from isolated strains of *N. gonorrhoeae* and the patients' swab samples using QIAamp DNA mini kit (Qiagen Sciences Inc., Germantown, MD, USA) according to the manufacturer's instructions.

2.5. Fabrication of the bioelectrode

Screen-printed gold electrodes (Au) procured from DropSens, Spain, were used for the study. Before self-assembled monolayer formation, the electrodes were treated with 50 μL of 0.5 mol/L H_2SO_4 solution containing 10 mmol/L KCl by cycling 10 times the potential between 0.0 and +1.7 V with a scan rate of 100 mVs^{-1} (Loaiza et al., 2008). The characteristic oxidation/reduction peaks were obtained at +1.35 V and +0.65 V, respectively. The thiolated probe DNA was activated using 0.1 mol/L dithiothreitol as per the manufacturer's instructions. The pre-treated electrodes were incubated overnight with 10 μL of 1 μmol/L activated probe in an inert atmosphere in humid chamber with a humidity of 80% to form self-assembled monolayer. The bioelectrodes were layered with 40 μL of 1 mmol/L MCH for 2 h to prevent any non-specific binding of DNA on the surface. The electrodes were thoroughly rinsed with deionized water and dried at each step. These electrodes were stored at 4 °C till further use. Fig. 1 depicts the graphic representation of the above-mentioned protocol. Electrochemical characterization of the bioelectrodes (Pristine gold electrode, electrode immobilized with probe DNA, and electrode hybridized with complementary DNA) was performed using cyclic voltammetry (CV), differential pulse voltammetry (DPV), and electrochemical impedance spectroscopy (EIS) in phosphate buffer saline electrolyte (PBS) (50 mmol/L, pH 7.0, 0.9% NaCl) containing 5 mmol/L $[Fe(CN)_6]^{3-/4-}$ (Potentiostat/Galvanostat; Autolab Eco Chemie, Utrecht, The Netherlands). Surface characterization was done using scanning electron microscopy (SEM, LEO-440).

2.6. Hybridization studies with synthetic DNA

The hybridization detection studies were performed on thssDNA-Au electrode by incubating with 10 μL complementary, non-

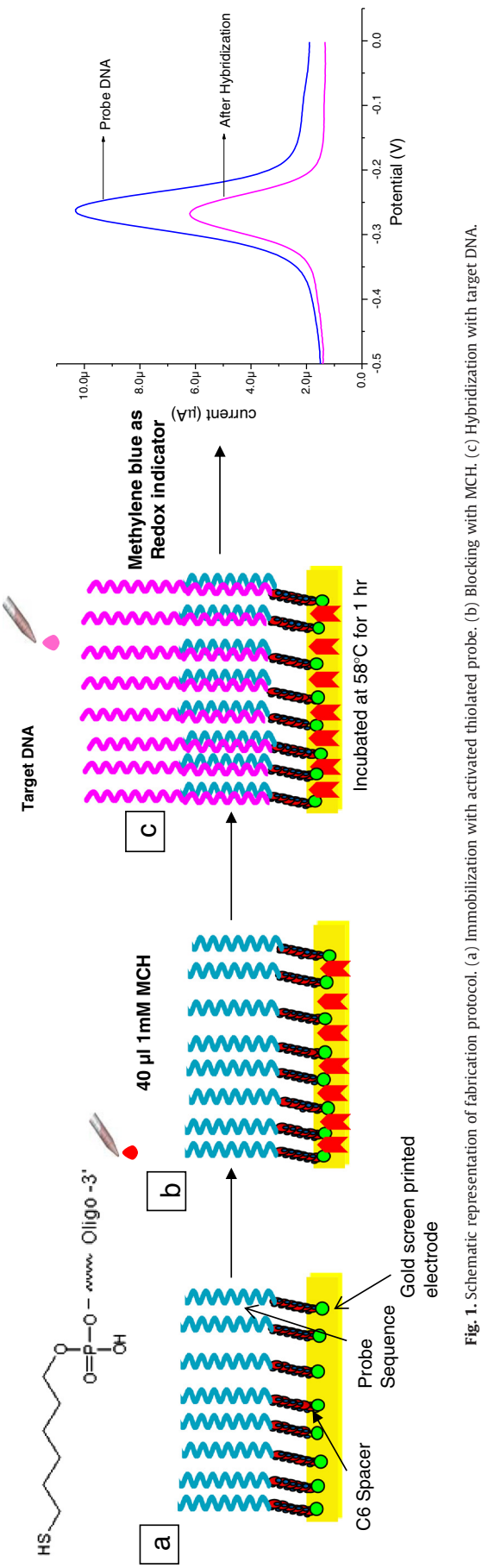


Fig. 1. Schematic representation of fabrication protocol. (a) Immobilization with activated thiolated probe. (b) Blocking with MCH. (c) Hybridization with target DNA.

complementary, and OBM DNA solution (0.1 µmol/L) in 5 mmol/L phosphate buffer, pH 7.0, containing 0.9% (w/v) NaCl, for 1 h at 58 °C. These were then rinsed with deionized water to remove any unbound DNA and air dried. Subsequently, DPV measurements of thssDNA-Au electrode treated with synthetic DNA were carried out after 20 µmol/L MB pre-treatment at +0.1 V for 10 s, in 5 mmol/L phosphate buffer pH 7.0 containing 0.9% (w/v) NaCl.

2.7. DNA amplification by PCR

DNA extracted from bacterial culture (*N. gonorrhoeae* standard strains and strains comprising of the SP panel) and patient samples were amplified by PCR (Verma et al., 2012). Sensitivity (SN) of the PCR assay was determined using serially diluted DNA of control strain *N. gonorrhoeae* (ATCC 49226). Aliquots of amplicons were prepared; one was used for electrophoretic analysis and the other for biosensor evaluation. The PCR products were visualized by electrophoresis on agarose gel (1.5% w/v). Agarose gel was prepared in 0.5× Tris-borate ethylenediamine tetraacetic acid buffer containing 0.5 µg/mL of ethidium bromide. Four microliters of PCR product was mixed with 6× gel loading buffer and loaded on the gel. Electrophoresis was performed at constant voltage of 5 V/cm until the dyes bromophenol blue and xylene cyanol migrated to the appropriate distance. The amplicons were visualized by Gel Doc™ (Bio-Rad, Hercules, CA, USA). A 188-bp amplified product was obtained for positive samples.

2.8. Hybridization studies with PCR amplified DNA from culture

Serial dilutions of amplified gonococcal DNA (from control strain) were prepared. Ten microliters of this diluted DNA was denatured at 95 °C for 5 min and then immediately chilled in ice to obtain single-stranded DNA (ssDNA). This processed DNA was then used for hybridization studies using the thssDNA-Au bioelectrode. The bioelectrode was also evaluated for its SP and diagnostic stability using amplified DNA from bacterial culture.

2.9. Hybridization studies with PCR amplified DNA from patient samples

Known positive and negative patient samples were amplified, and this amplified DNA was then used for evaluation of the thssDNA-Au bioelectrode. The amplicons were diluted 10^{-6} times (based on the reportable range of the bioelectrode) and then denatured as mentioned earlier. The bioelectrode was incubated with 10 µL of the denatured product and DPV measurements carried out after 20 µmol/L MB pre-treatment at +0.1 V for 10 s, in 5 mmol/L phosphate buffer pH 7.0 containing 0.9% (w/v) NaCl.

2.10. Statistical analysis

The results of electrochemical hybridization response of the bioelectrode with test samples (synthetic DNA/amplicons) in presence of MB were reported as percentage reduction in peak current (I_{PA}), calculated as per Eq. 1. These variables were used for determining the SN and SP associated with the individual cut-off values.

$$\text{percentage difference} = \frac{\text{peak current of probe} - \text{peak current of test}}{\text{peak current of probe}} \times 100 \quad (1)$$

ROC curve was plotted using the data analysis and statistical software STATA® version 9 (StataCorp, College Station, TX, USA) to determine the ability of the bioelectrode to accurately distinguish positives from negatives. SN, SP, positive predictive value (PPV), and negative predictive value (NPV) of the biosensor were calculated with culture as gold standard. The clinical utility index (CUI) and Youden

index (J) (Caraguel et al., 2011; Mitchell, 2011) were also determined for the bioelectrode as per Eqs. 2, 3, and 4.

$$J_c = SN_c + SP_c - 1; \text{ where c is the cut-off point} \quad (2)$$

$$\text{Positive clinical utility index (CUI+)} = \text{Sensitivity} \times \text{PPV} \quad (3)$$

$$\text{Negative clinical utility index (CUI}-\text{)} = \text{Specificity} \times \text{NPV} \quad (4)$$

3. Results

3.1. Electrophoretic analysis of PCR amplicons

A 188-bp band was observed on 1.5% agarose gel for positives. No band was obtained with other gram-negative bacteria or non–*N. gonorrhoeae Neisseria* species. The analytical SN of PCR was found to be 10^{-17} M (10^{-12} g) of DNA when detected by gel electrophoresis (Verma et al., 2012).

3.2. Surface characterization by SEM

Fig. 2 shows SEM images of bare Au electrode and thssDNA-Au electrode. The bare electrode shows a smooth morphology (Fig. 2a). After thssDNA immobilization, extensive network type morphology is observed (Fig. 2b).

3.3. Electrochemical characterization of the bioelectrode

Au, thssDNA-Au, and double-stranded DNA (dsDNA) Au (modified with probe–complementary DNA hybrid) were characterized electrochemically using CV in PBS (50 mmol/L, pH 7.0, 0.9% NaCl) containing 5 mmol/L $[Fe(CN)_6]^{3-/4-}$. After immobilization of thssDNA on pristine gold electrode (Fig. 3a), a decrease in the peak current was observed that is in accordance with our earlier study on gold plates (Singh et al., 2010). A further decrease in the peak current was observed after hybridization of the probe with the complementary DNA.

Fig. 3b shows the Nyquist plot of the EIS measurements carried out as a function of frequency on Au, thssDNA, and dsDNA hybridized with complementary DNA in 50 mmol/L PBS, pH 7.0 containing 5 mmol/L $[Fe(CN)_6]^{3-/4-}$. The charge transfer resistance (R_{ct}) of pristine gold electrode was found to be 0.199 kΩ. When thssDNA was immobilized onto pristine gold electrode, R_{ct} of the electrode increases to 0.953 kΩ that may be due to the presence of electro-negative phosphate skeleton that perhaps prevented $[Fe(CN)_6]^{3-/4-}$ ions from reaching the electrode surface for electron transfer during redox reaction. This implies that thssDNA was successfully immobilized onto the pristine gold electrode. Further increase in R_{ct} (3.512 kΩ) may be due to the negatively charged surface of the gold electrode hybridized with complementary DNA that prevented $[Fe(CN)_6]^{3-/4-}$ in solution from clustering on the electrode surface for electron exchange.

3.4. Response studies of the bioelectrode in presence of MB

Fig. 4a shows redox peaks of MB in presence of complementary, non-complementary, and OBM DNA. The reduction in peak current was interpreted in terms of percentage decrease and has been tabulated (Table 1). A decrease in peak current was observed only with complementary DNA and not for non-complementary and OBM DNA.

Fig. 4b shows variation of the electrochemical response of MB in presence of different dilutions (10^{-5} M to 10^{-22} M) of PCR amplicons obtained from the standard strain ATCC 49226 of *N. gonorrhoeae*. The peak current has been found to exhibit a linear correlation to the log [target concentration] in the range 1×10^{-9} M to 1×10^{-15} M (Fig. 4c) and obey Eq. (5).

$$\text{MB oxidation current } I_\mu A = -0.55\,[\text{DNA concentration}] + 14.11 \quad (5)$$

with regression coefficient of 0.979. However, when further dilutions were tested, an increase in the peak current was observed with increasing dilution. A linear correlation was observed between the peak current and the log [target DNA concentration] in the range 1×10^{-15} M to 1×10^{-22} M (Fig. 4d) obeying Eq. (6).

$$\text{MB oxidation current } I_\mu A = 1.18\,[\text{DNA concentration}] - 12.8 \quad (6)$$

with regression coefficient 0.932. A significant decrease in the redox peak has been observed up to 10^{-20} M corresponding to 10^{-19} g of PCR product, recorded as the detection limit of the assay. The reportable range, i.e., the range that gives positive results for the bioelectrode was 10^{-9} M to 10^{-20} M. The experiments have been performed in triplicate. SP studies demonstrate that the bioelectrode is highly specific for *N. gonorrhoeae* showing a significant decrease in MB peak current only with gonococcal amplicon (Figure not shown).

3.5. Evaluation of bioelectrode

The DNA concentration of the patients' amplicons was determined spectrophotometrically. Based on this, the amplicons were diluted 10^{-6} times to bring them within the reportable range. Fig. 5a shows the performance of the bioelectrode with respect to amplicons of clinical samples. The reduction in peak current was interpreted in terms of the percentage decrease. The individual percentage decrease in current for

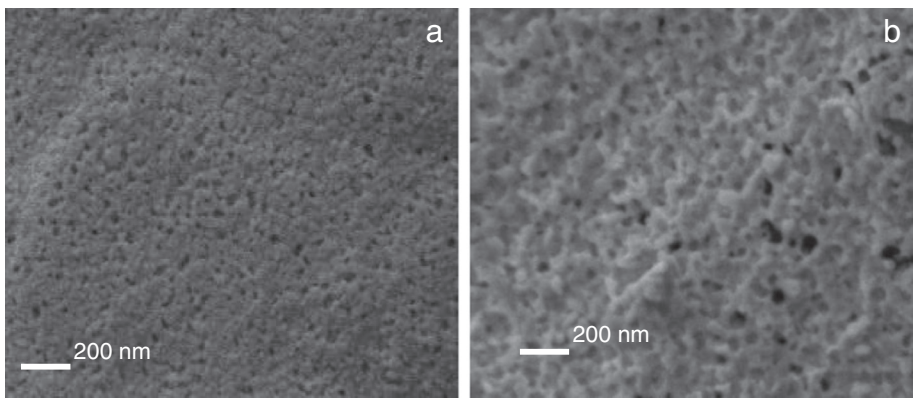


Fig. 2. Scanning electron microscopy. (a) Pristine Au electrode, (b) thssDNA-Au electrode.

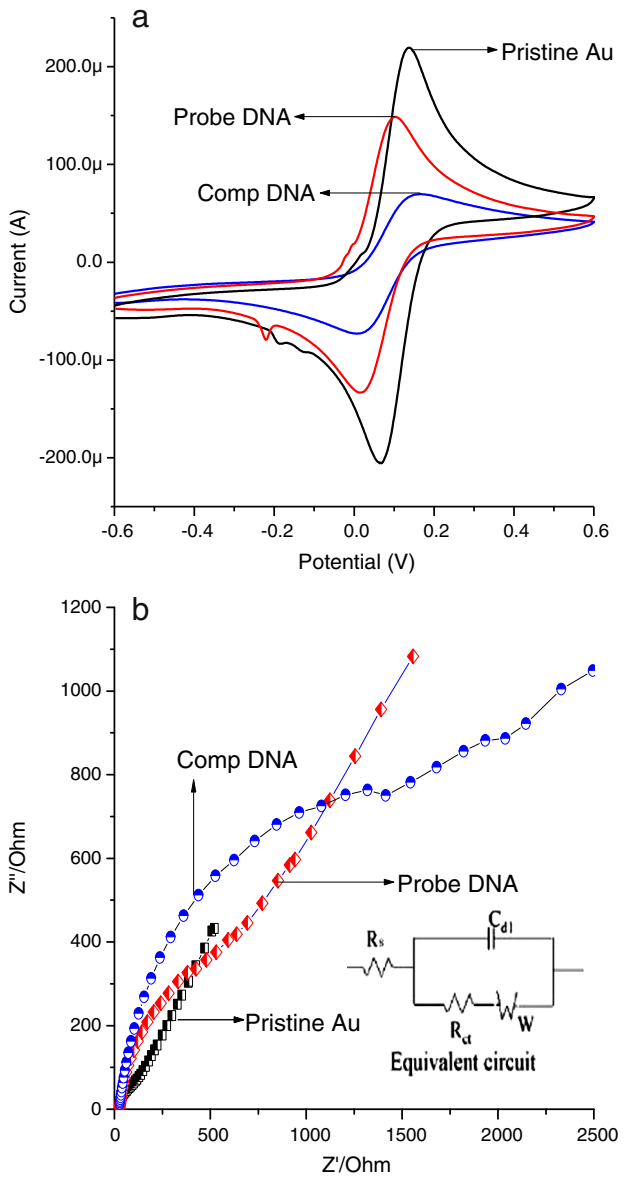


Fig. 3. Electrochemical characterization of bioelectrode. (a) CV studies of probe and complementary DNA in PBS of pH 7.0 containing 5 mmol/L $[Fe(CN)_6]^{3-/4-}$. (b) Electrochemical impedance spectroscopic studies of probe and complementary DNA in PBS of pH 7.0 containing 5 mmol/L $[Fe(CN)_6]^{3-/4-}$.

each of the test samples, and the corresponding SN and SP of the test assay taking each of these points as cut-off were tabulated (Table 2A). The optimal cut-off according to Youden index is 11.49% decrease in the signal intensity. Samples giving 11.49% decrease in current or more were considered as positives. This is in accordance with the ROC graph (Fig. 5b). The area under the curve was found to be 0.9706 indicating that the test (biosensor) has an excellent accuracy for distinguishing the positive/negative amplicons of *N. gonorrhoeae*. The SN, SP, PPV, and NPV of the biosensor assay was 96.2%, 88.2%, 92.6%, and 93.8%, respectively, when compared to culture, which is the "gold standard" (Table 2B). The CUI (+) was 0.89 and CUI (−) was 0.83, rating the test as excellent (Mitchell, 2011).

4. Discussion

It is estimated that the complications associated with gonorrhoea may perhaps be responsible for loss of more than 250,000 disability adjusted life years each year, establishing its economic burden and hence the need for prompt diagnosis (Bala et al., 2010). Molecular detection techniques for *N. gonorrhoeae* based on Nucleic acid amplification tests, predominantly PCRs, are being developed and evaluated by laboratories worldwide (Geraats-Peters et al., 2005; Whiley et al., 2006). However, difficulties regarding SP have been experienced, with many of these systems producing false positives with non-pathogenic *Neisseria* species (Smith et al., 2005). Further, the performance of these tests in case of asymptomatic patients is not known for most settings. Nowadays, there is a great interest in biotechnological developments delivering highly specific, reproducible, and low-cost screening methods for the detection of pathogens. Thus, the use of biosensor has been proposed as a means of obtaining rapid positive or negative results from a sample. This study was undertaken in line with such developments wherein an electrochemical DNA biosensor for detection of *N. gonorrhoeae* was developed.

MB is a commonly used electrochemical indicator for DNA biosensor, and it was selected for our experiments to detect DNA hybridization. MB produced different electrochemical responses on interaction with ssDNA and dsDNA. Both showed a decrease in the redox peak current as compared to that of the bare gold electrode, but the extent of decrease, however, varied. A sequential decrease was observed with bare gold electrode, probe immobilized electrode (ssDNA), and electrode hybridized with target DNA (dsDNA). This is in accordance with the previously published reports using MB as hybridization indicator (Erdem et al., 2001; Meric et al., 2002; Tichoniuk et al., 2008; Yang et al., 2002).

When the bioelectrode was tested using synthetic DNA, a decrease in MB redox current was observed with complementary DNA only and not for non-complementary and OBM DNA. It appears that the ssDNA and the dsDNA perhaps interact with MB with different binding mode. MB interacts with ssDNA by electrostatic binding, while the effect of electrostatic and intercalation binding coexists in dsDNA (Gu et al., 2002). Another hypothesis put forward by Gu et al. (2002) states that the decrease in current with dsDNA may perhaps result from the decrease of free MB concentration in buffer due to the formation of MB-ssDNA or MB-dsDNA. MB gets intercalated within the dsDNA, and hence, it may not be freely available, causing decrease in the signal intensity.

The DNA biosensor was further used for the detection of 188-bp PCR product of the *opa* gene of *N. gonorrhoeae*. This conserved multicopy (11–13) gene of *N. gonorrhoeae* is an attractive diagnostic target. It has been reported that terminal sequences give better exposition and, hence, better hybridization for detection of PCR amplicons as compared to the internal sequences (Gabig-Ciminska et al, 2004). Keeping this in view, the 19-mer oligonucleotide sequence, which was used as the forward primer for the *opa*-based PCR, has been used as the probe for biosensor studies. This was also observed to give a better performance as compared to the 20-mer probe (Singh et al., 2010) used in the earlier studies (data not shown).

The bioelectrode was found to be highly sensitive with a detection limit of 10^{-20} M (10^{-19} g) of PCR product and a reportable range of 10^{-9} M to 10^{-20} M, whereas the use of gel electrophoresis to detect the PCR products provided an SN of picograms of DNA without any SP check as detection is based only on the observed band size. Amplification of signal by the electrochemical biosensor in addition to the DNA amplification provided enhanced SN to the assay apart from obviating the need for toxic chemicals.

The fabricated bioelectrode when evaluated using known positive and negative amplicons of patients' samples was found to be highly specific. A decrease in current was obtained for positives, while negatives either clustered around probe or an increase in current was observed. Increase in the peak height may be attributed to non-specific adsorption of DNA amplicons on the matrix. Similar results with PCR amplicons have been reported in literature (Meric et al., 2002). A threshold/cut-off was determined for the bioelectrode by plotting a ROC curve. The ROC curve for the biosensor was seen to lie far away from the "chance diagonal line",

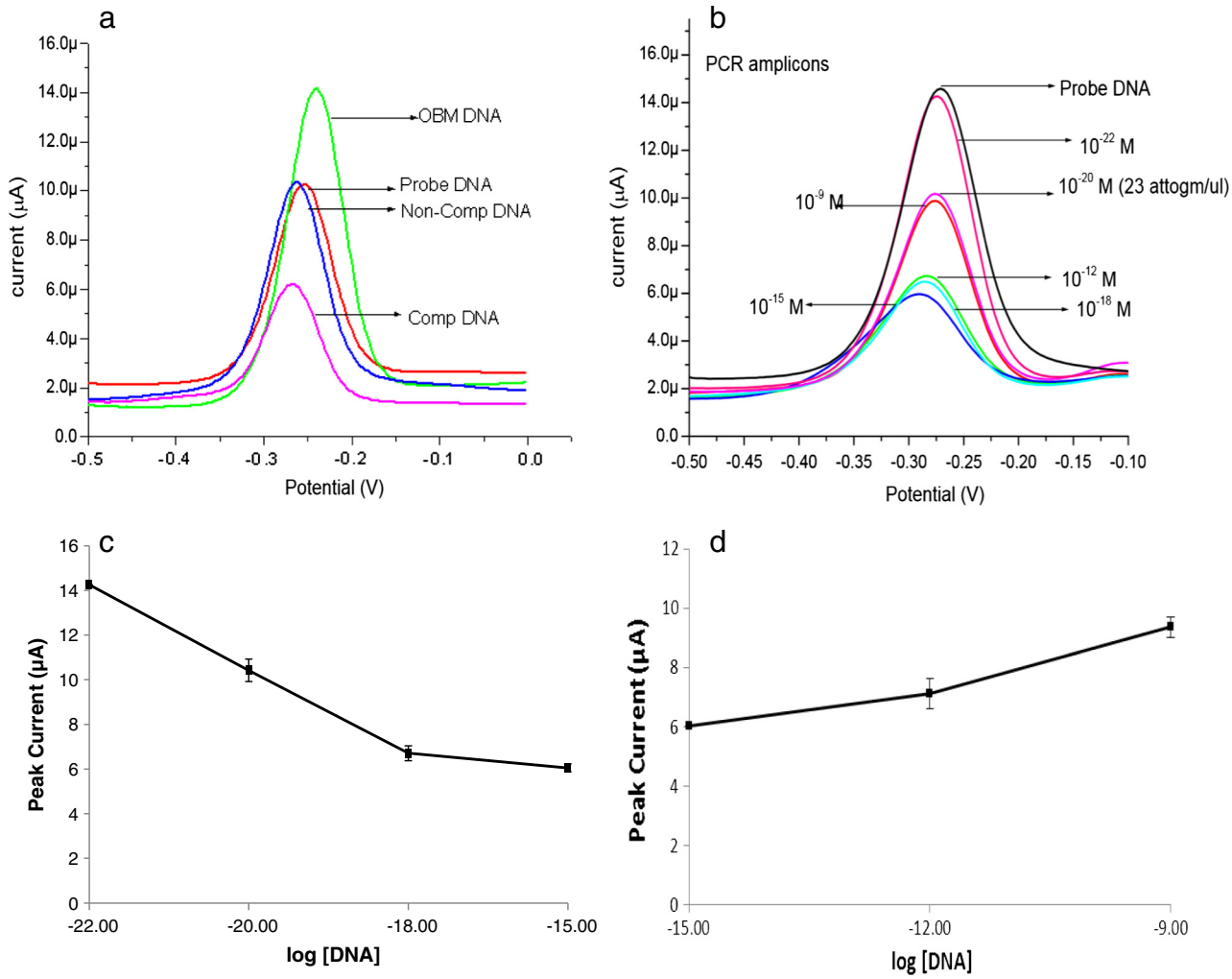


Fig. 4. Response studies of MB. (a) Differential pulse voltammogram of synthetic DNA after 20 µmol/L MB pretreatment at +0.1 V for 10 s, in 0.05 mol/L phosphate buffer pH 7.0 containing 0.9% NaCl. (b) Differential pulse voltammogram of different dilutions of PCR amplicons after 20 µmol/L MB pretreatment at +0.1 V for 10 s, in 0.05 mol/L phosphate buffer pH 7.0 containing 0.9% NaCl. (c) Linear relationship between peak current and log [DNA concentration] in the range 1×10^{-15} M to 1×10^{-22} M. (d) Linear relationship between peak current and log [DNA concentration] in the range 1×10^{-9} M to 1×10^{-15} M.

showing a high SN along with a high SP. The area under the curve was found to be 0.9706 indicating that the test (biosensor) has an excellent accuracy for distinguishing the positive/negative amplicons of *N. gonorrhoeae* (Weinstein et al., 2005; Zou et al., 2007). The optimal threshold in terms of percentage decrease on the basis of ROC analysis was determined as 11.49% thereby implying that amplicons exhibiting 11.49% decrease in current or more were considered as positives. However, the accuracy and consistency of this threshold need to be further assessed and validated with a larger number of samples.

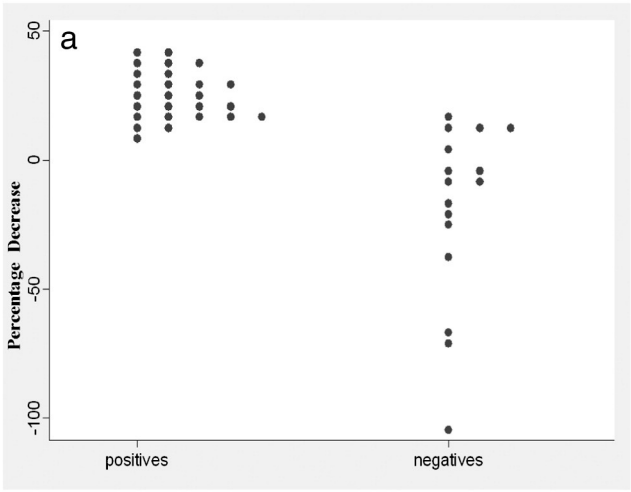
Table 1
Percentage decrease in current of thssDNA-Au electrode after treatment with complementary, OBM, and non-complementary DNA sequences.

Electrode	MB redox current (µA)	Percentage decrease in current[a]
thssDNA-Au	10.3	-
thssDNA-Au with complementary DNA	6.2	40
thssDNA-Au with OBM	14.1	−35.7
thssDNA-Au with non-complementary DNA	10.3	0

[a] Calculated w.r.t. current read for probe modified electrode.

Biosensors are increasingly being evaluated for detection of pathogens in environmental samples following different amplification strategies (direct PCR, asymmetric PCR). However, limited reports are available for application of biosensors for detection of pathogens in patient samples (Kerman et al., 2004; Meric et al., 2002; Pedrero et al., 2011). In majority of the cases, representative sample is used, which is often not sufficient to determine the performance of the bioelectrode (Kobayashi et al., 2004; Meric et al., 2002). In the present study, the use of fabricated gold electrode with MB for electrochemical detection of hybridization has demonstrated an efficient strategy for sensitive and specific detection of PCR amplified products obtained from *N. gonorrhoeae* culture isolates and patient samples. This strategy offers a low detection limit, and it may be particularly useful for detection of asymptomatic gonococcal infections wherein the bacterial load is low. This may provide enhanced diagnosis and hence greatly improve gonorrhoea control efforts. Further, given the advantages provided by this kind of sensor and no need of expensive optics or high-voltage power supplies, it could be readily considered for the development of portable PCR micro-devices directed to rapid detection of this pathogen.

The integration of biosensors with microfluidics may permit development of lab-on-a-chip systems, which could efficiently reduce the turnaround time of the diagnostic cycle. Microfluidic PCRs are widely being explored for development of POCTs. Integration of chip-



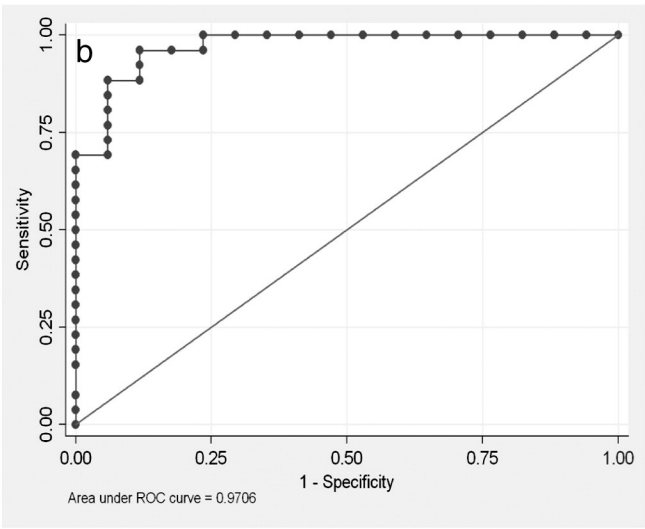


Fig. 5. (a) Dot plot of positive and negative patient samples. Readings plotted as percentage decrease in signal intensity for DPV measurements after 20 μmol/L MB pretreatment at +0.1 V for 10 s, in 0.05 mol/L phosphate buffer pH 7.0 containing 0.9% NaCl. (b) ROC curve analysis of the bioelectrode.

based detection system would provide a highly sensitive and specific pathogen detection tool. Our future work is focused on the application of the fabricated bioelectrode directly on clinical samples.

Table 2A
Detailed report of SN, SP, and associated Youden index.

Percentage decrease in peak current	Positive samples		Negative samples		SN	SP	Youden index
	TP	FN	FP	TN			
≥5.92 and below	26	0	≥5	≤12	100.0%	≤70.6%	≤.706
≥8.86	26	0	4	13	100.0%	76.5%	.765
≥11.28	25	1	4	13	96.2%	76.5%	.726
≥11.31	25	1	3	14	96.2%	82.4%	.786
≥11.49	25	1	2	15	96.2%	88.2%	.844
≥11.93	24	2	2	15	92.3%	88.2%	.805
≥13.85	23	3	2	15	88.5%	88.2%	.767
≥15.09	23	3	1	16	88.5%	94.1%	.826
≥15.11	22	4	1	16	84.6%	94.1%	.787
≥15.53	21	5	1	16	80.8%	94.1%	.749
≥16.02	20	6	1	16	76.9%	94.1%	.710
≥16.4	19	7	1	16	73.1%	94.1%	.672
≥16.75	18	6	1	16	69.2%	94.1%	.633
≥20.35 and above	≤17	≥7	0	17	≤69.2%	100.0%	≤.692

TP = True positives; FN = False negatives; FP = False positives; TN = True negatives.

Table 2B
Diagnostic indices of biosensor.

	Positive	Negative	SN (95% CI)	SP (95% CI)	PPV (95% CI)	NPV (95% CI)	ROC area (95% CI)
≥11.49%	25	2	96.15% (78.41–99.79%)	88.24% (62.28–97.93%)	92.59% (74.28–98.70%)	93.75% (67.71–99.67%	0.97 (0.93–1.00)
≤11.49%	1	15[a]					

$P < 0.05$[b], statistically significant

[a] Fourteen negatives and 1 blank.
[b] Median percentage decrease in current read for positive sample was significantly different from the negative with the $P < 0.001$.

5. Conclusions

In this manuscript, an *opa*-gene–based electrochemical DNA biosensor has been constructed and applied to synthetic DNA and PCR amplified products from culture isolates/standard strain of *N. gonorrhoeae* and known positive and negative samples. The electrochemical behaviour of MB has been investigated, and the accuracy of the biosensor in distinguishing positive and negative amplicons has been determined. The coupling of electrochemical detection with PCR amplification shows the advantage of higher SN and increased SP for the detection of *N. gonorrhoeae*. This may prove to be particularly valuable for the identification of asymptomatic infections thereby contributing to gonorrhoea control.

6. Limitations

With the existing biosensor, pre-amplification of samples by PCR is required for accurate detection of *N. gonorrhoeae* in clinical samples. Additionally, we observed a high background noise with the negatives, which may have concealed the signal decrease with positives leading to false results. Therefore, further modifications are required for improving the matrix. Use of a different blocking agent(s) for the matrix and a modification in the detection strategy like use of capture-probe and detector-probe may provide better results.

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