Self-assembled monolayer based electrochemical nucleic acid sensor for *Vibrio cholerae* detection

Manoj K Patel 1, 2, Pratima R Solanki1, Sachin Khandelwal4, Ved V Agrawal1, S G Ansari2 and B D Malhotra3
1Department of Science and Technology Centre on Biomolecular Electronics, Biomedical Instrumentation Section, National Physical Laboratory (CSIR), Dr. K. S. Krishnan Marg, New Delhi -110 012, India
2Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi 10 025, India
3Department of Biotechnology, Delhi Technical University, Shahbad Daulatpur, Main Bavana Road, Delhi 110 042, India
4Microbiology Division, National Centre for Disease Control, 22, Sham NathMarg, Civil Lines, New Delhi 110 054, India

E-mail: bansi.malhotra@gmail.com (B D Malhotara)

Abstract. Nucleic acid sensor has been fabricated by immobilization of thiolated (5’ end) single stranded deoxyribonucleic acid probe (ssDNA-SH) onto gold (Au) coated glass electrode for *Vibrio cholerae* detection. This ssDNA-SH/Au bioelectrode characterized using atomic force microscopy (AFM), Fourier transforms infrared spectroscopy (FT-IR) and electrochemical technique, has been used for hybridization detection of genomic DNA (dsDNA/Au). This ssDNA-SH/Au bioelectrode can specifically detect up to 100-500 ng/µL genomic DNA of *Vibrio cholerae* within 60 s of hybridization time at 25°C by cyclic voltammetry (CV) using methylene blue (MB) as electro-active DNA hybridization indicator. The value of sensitivity of the dsDNA/Au electrode has been determined as 0.027µA/ng cm⁻² with regression coefficient as 0.978. This DNA bioelectrode is stable for about 4 months when stored at 4°C.

1. Introduction
Cholera is endemic in southern Asia and parts of Africa and Latin America, where seasonal outbreaks occur widely and are particularly associated with poverty and poor sanitation. [1-2]. It is an acute intestinal infection caused by ingestion of food or water contaminated with the bacterium *Vibrio cholerae*, which is a Gram-negative bacterium that produces cholera toxin (CT), an enterotoxin, whose action on the mucosalepithelium lining of the small intestine is responsible for the disease’s most salient characteristic, exhaustive diarrhea [3-4]. Most of the strains of *V. cholerae* are non-virulent but O1 and O139 is responsible for disease[5-6]. As compared to O139 antigen, O1 antigen has been found in majority of patients affected by *V. cholerae* which curved-rod shaped and oxidase-positive bacterium [7].
Many biomolecules such as gangliosides, antibodies, cholera toxin and somatic antigens (O1 and O139) have been used for *V. cholerae* detection. CT has become a very important compound in detecting bioterrorism, and there has been increasing interest in the development of rapid and sensitive methods for the determination of CT [8]. The conventional methods for *V. cholerae* detection include enzyme linked immunosorbent assay/ immunological detection kits, southern hybridization and polymerase chain reaction [6, 9-11]. However, all these methods are time-consuming, expensive and require expertise [12]. There is increased interest towards the application of biosensors for fast, low-cost, sensitive and reliable detection of *V. cholerae*. The deoxyribonucleic acid (DNA) biosensors are potentially useful for clinical applications to determine the pathogenic bacteria [13].

Biological macromolecules, such as nucleic acids, both (DNA) and ribonucleic acid (RNA) are known to play an important role towards the rapid development of molecular genomics, biotechnology and medical diagnostics [14]. Nucleic acid based biosensors based on the hybridization technique are gaining more attention over traditional diagnostic methods as they are faster, simpler and economical [15-17]. Several techniques have been used for DNA detection including fluorescence detection, mass spectrometry, electrochemical, surface plasmon resonance spectroscopy and quartz crystal microbalance [18]. Besides this, an electrochemical detection technique has advantages like simplicity, speed, low-cost, high sensitivity and specificity as compared to molecular detection approaches [19].

Self-assembled monolayers (SAMs) have been used in electro analytical chemistry due to their uniqueness in terms of well-ordered structure, ease of preparation, flexibility, reproducibility, organization, molecular control and low non-faradaic currents etc. [20-23]. The thiol groups chemisorbed onto the gold surface via the formation of a gold thiol bond [24-25] to produce a densely packed, highly ordered monolayer. The self-assembly process is mediated through non-specific adsorption, electrostatic interactions, covalent bonding and specific interactions. The SAMs are spontaneously formed by the adsorption/binding of organic molecules from solution or the gas phase onto a solid-state surface [26]. Zhao et al. have utilized hydroxyl-terminated SAM for covalent immobilization of dsDNA on gold surface [27]. SAM based [28] surface plasmon [29] and piezoelectric [30] immunosensors have been used for detection of *Salmonella Typhimurium, Leukemia* and *Escherichia coli* (E. coli). Prabhakar et al. have reported 5’ thiol end labeled DNA probe for detection of *M. tuberculosis* [31].DNA biosensor based on electrochemical hybridization using methylene blue as an indicator has been reported for detection of Chronic Myelogenous Leukemia, Human apolipoprotein E, *Bar Gene, E.coli*, etc. using glassy carbon electrode, graphite screen printed electrode, stearic-acid-modified carbon paste electrode, polyaniline, respectively [32-34].

We report the results of the immobilization of 5'-thiol end labeled DNA probe (23 bases) and hybridization with genomic DNA of *Vibrio cholerae* on Au bare surface were studied using Methylene blue (MB) as an electroactive indicator.

2. Materials and methods

2.1. Chemicals and reagents

Tris (hydroxymethyl) amino methane, ethylene diamine tetra acetic acid (EDTA), sodium dihydrogen ortho-phosphate and *di*-sodium hydrogen orthophosphate were procured from Qualigens, India and Methylene blue (MB) was procured from Sigma-Aldrich, USA. HPLC purified 23 bases thiolated modified oligomer probe (ssDNA-SH) 5’-HS - GCA TAT GCA AAT GGA ACA CCT CA- 3’ were procured from GenxBio Health Sciences Pvt. Ltd, India. All reagents were prepared in de-ionized water (Milli Q 10 TS) and solutions were autoclaved prior to being use. DNA solutions were prepared in TE buffer (10 mMTris, 1mM EDTA, pH 8.0). All Bacterial isolates and genomic DNA sample were obtained from patients at the National Centre Diseases Control (NCDC), New Delhi, India. Bacterial isolates from feces were cultured on Thiosulfate Citrate Bile Salts Sucrose Agar (*TCBS* Media) and grown at 35°C for 24-48 h and the combination of alkaline peptone water.
2.2. Fabrication of ssDNA-SH/Au bioelectrode

Gold coated glass electrodes were first cleaned with piranha solution (H_2SO_4 and H_2O_2 in 7:3 ratio) for 5 min. After dipping in 70% ethanol for 2-3 min, the electrode was washed with water and dried at room temperature. The thiolated DNA probe (ssDNA-SH) 6.6 ng/10 µl was then immobilized onto gold electrode area (0.5 cm^2) and kept for 24 h at 25°C. The unbound probe was removed by several washings with TE buffer and dried. Genomic DNA (dsDNA) was hybridized (after denaturation at 95°C) for 5 min with immobilized probe onto gold electrode surface. After hybridization the electrode was washed thrice with buffer to remove the unbound ssDNA. The schematic shows the immobilization and hybridization procedure in Scheme 1.

Scheme 1. Schematic representation shows the synthesis of SH labeled probe from SH molecule and hybridization of genomic DNA (dsDNA) with immobilized probe.

2.3. Sensor characterization

The surface morphology of ssDNA-SH/Au electrode and dsDNA/Au electrode were delineated using atomic force microscopy (VEECO, USA) in non-contact mode. Electrochemical experiments were carried out with a Electrochemical analyzer (Model AUT-84275, Auto lab) using three-electrode system containing gold coated glass electrode as working, Ag/AgCl as reference and platinum as counter electrode. MB (40 µM) was used as redox indicator in phosphate buffer saline (PBS, 0.05 M, pH 7.0, 0.9% NaCl). The cyclic voltammetric experiments were performed at 20 mV/s in PBS buffer at 25°C. Contact angle (Dataphysics, Model OCA15EC) measurement was done by sessile drop method before and after the immobilization/hybridization of DNA.

3. Results and discussion

3.1. AFM studies

Figure 1A. shows results of morphological studies obtained using AFM of bare Au electrode (image a), ssDNA-SH/Au electrode (image b) and dsDNA/Au [hybridization with genomic DNA(image c)] after denaturation at 95°C. Image (a) shows the nonporous morphology with uniform distribution of granular structure with roughness 6.68 nm (Fig.1Aa) and after the probe immobilization the roughness increases (18.4 nm) due to formation of well aligned self-assembled monolayer of thiolated ssDNA fabrication onto Au surface (Scheme 1) that filled the all the pores present onto Au surface. However,
the surface morphology after the hybridization with genomic DNA (image c) has been observed as 16.3 nm that indicate that genomic DNA hybridized with the DNA probe present onto gold surface.

3.2. Contact angle measurements

Contact angle measurements (Sessile drop method) have been carried out to investigate the formation of self-assembled monolayer onto Au surface. Contact angle studies have been carried out using deionized distilled water to characterize the immobilization of probe (ssDNA) and after hybridization with genomic DNA (dsDNA) onto the gold surface (Fig.1B). The change in the value of the contact angle reveals the hydrophobic/hydrophilic character of the surface due to immobilization of thiolated probe and hybridization with genomic DNA molecules. The contact angle value of the bare gold film (image a) is found as 76.46° and decreases to 55.56°after the immobilization of thiolated ssDNA probe (image b) and further decreases to 55.33°after DNA hybridization (image c). The decrease in the contact angle values can be attributed to the presence of hydrophilic group (OH) in DNA probe. These results indicate successful binding of the nucleic acid (probe/DNA) onto Au electrode surface.

**Figure 1A.** AFM micrographs of bare Au electrode (image a), (image b) after immobilization with thiolabeled probe (ssDNA - SH/Au) and (image c) after hybridization with genomic DNA (dsDNA-SH/Au) onto Au surface.

**Figure 1B.** Contact angle measurements of (a) bare Au electrode (76.46°) (b) immobilization ssDNA - SH/Au (55.56°) and (c) dsDNA-SH/Au (55.33°) on gold coated glass electrode.
3.3. **FT-IR studies**

The FT-IR spectra of (a) ssDNA-SH/Au bioelectrode (b) dsDNA/Au exhibits peak at 891, 1047, 1215, 1392, 1461, 1455, 1515, 1548, 1650, 1903, 2066, 3261 cm\(^{-1}\) corresponding to the functional groups of DNA (Fig. 2). The peaks found at 891 to 1215 cm\(^{-1}\) correspond to asymmetric and symmetric PO\(_4\)\(^{3-}\) groups of the phosphodiester-deoxyribose backbone. The peaks seen at 1455, 1515, 1548 and 1650 cm\(^{-1}\) correspond to cytosine, adenine, thymine and guanine respectively. A FT-IR spectrum after hybridization with both amplified and genomic DNA shows similar peaks of different bases with increased peak intensity. The peaks seen at 1515, 1548 and 3261 cm\(^{-1}\) are attributed to N-H bending and stretching of DNA bases purine and pyrimidine ring [35]. The 1515 - 1650 cm\(^{-1}\) peaks correspond to C=O, C=N, C=C stretching and exocyclic -NH\(_2\) bending vibrations in the DNA bases. These results indicate the immobilization of DNA probe onto Au surface and hybridization with genomic DNA.

![FT-IR spectra of (a) ssDNA - SH/Au and (b) dsDNA-SH/Au of *Vibrio cholerae*.](image)

**Figure 2.** FT-IR spectra of (a) ssDNA - SH/Au and (b) dsDNA-SH/Au of *Vibrio cholerae*.

3.4. **Electrochemical impedance studies**

![Impedance spectra of (a) bare Au electrode (b) ssDNA - SH/Au and (c) dsDNA-SH/Au of O1 gene in PBS containing 5 mM [Fe(CN)\(_6\)]\(^{3-4}\).](image)

**Figure 3.** Impedance spectra of (a) bare Au electrode (b) ssDNA - SH/Au and (c) dsDNA-SH/Au of O1 gene in PBS containing 5 mM [Fe(CN)\(_6\)]\(^{3-4}\).
Figure 3 exhibits the Faradic impedance spectra in the frequency range 0.01-10^5 Hz, presented by the Nyquist plots of (a) bare gold (b) ssDNA-SH/Au and (c) dsDNA/Au in PBS containing 5 mM [Fe(CN)_6]^{3-4}. The value of Rct, charge-transfer resistance, obtained as 3.75 x 10^2 Ω (curve a) for bare gold is lower than that of ssDNA-SH/Au electrode. This smaller semicircle diameter indicates faster electron-transfer kinetics of [Fe(CN)_6]^{3-4} on bare Au electrode (curve a). However, after the immobilization of ssDNA-SH (curve b), Rct value increases (1.09 x10^3 Ω) due to presence of electronegative phosphate skeletons that perhaps prevent [Fe(CN)_6]^{3-4} ions from reaching the electrode surface for electron transfer during redox reaction. This reveals that ssDNA-SH has been immobilized onto Au surface. Further, increase in the Rct value after hybridization of probe with the genomic DNA (6.74 x 10^2 Ω; curve c) can be attributed to the increased concentration of negatively charged backbone of DNA onto the surface of the electrode that may cause repulsion between the similarly charged redox couple of the system. The increase in the Rct value indicates duplex form of DNA indicating immobilization of ssDNA hybridization with genomic DNA.

3.5. Cyclic voltammometric studies

Figure 4A shows results the cyclic voltammetric (CV) carried out for (a) bare gold (b) ssDNA/Au electrode and (c) dsDNA-SH/Au (500 ng) electrode in PBS containing methylene blue (40 µM). Methylene blue (MB) is an intercalating organic compound has been used for electrochemical DNA detection as their response changes upon DNA hybridization. It can be seen that the oxidation peak current for bare gold electrode is higher (6.80 µA, curve a) than that of ssDNA-SH/Au electrode (0.74 µA, curve b), indicating immobilization of the DNA probe onto Au surface. The higher oxidation peak current of bare Au surface as compared to that of the ssDNA-SH/Au electrode, is attributed to the enhanced flow of electrons between the gold electrode and MB. On the other hand, the oxidation peak current of dsDNA-SH/Au bioelectrode (4.64 µA, curve c) increases as compared to that of the ssDNA-SH/Au bioelectrode (curve b). It appears that dsDNA-modified surface is more favorable for MB molecules to undergo redox reaction than bare gold surface, indicating a pre-concentration of MB on the DNA modified surface due to intercalation. The sharp cathodic peak also indicates that MB underwent redox through the intercalated state [36]. Moreover, the highest MB oxidation signal was obtained at the electrode hybridization with genomic DNA sequence (curve c), indicating that the

![Figure 4A](image-url)

**Figure 4A** Cyclic voltammogram of (a) bare Au electrode (c) dsDNA-SH/Au bioelectrode and (b) ssDNA-SH/Au electrode 500 ng at 20 Vs\(^{-1}\) in PBS containing 40 µM MB and (B) Cyclic voltammogram of ssDNA-SH/Au bioelectrode on increasing scan rate from 10 to 90 at 20 Vs\(^{-1}\) in PBS (50 Mm, pH 7 containing 0.9 % NaCl) in 40 µM MB.
largest attachment of MB occurred at this electrode because of the strong affinity of MB with the formed dsDNA. The peak current (curve b) of MB decreases after the ssDNA probe [37]. This result can be perhaps be due to stronger association of MB molecules with unpaired nitrogenous bases of ssDNA-SH/Au bioelectrode (curve c), especially with guanine bases as compared to dsDNA. The ssDNA-SH/Au bioelectrode consists of the limited number of unhybridized nitrogenous bases that interact with MB, while genomic DNA comprises of prolonged unhybridized ssDNA sequence consisting of uncountable nitrogenous bases, resulting in increased MB molecules interaction. Thus oxidation current is found to be increased in case dsDNA-SH/Au bioelectrode as compared to that of the ssDNA-SH/Au electrode. The magnitude of the peak current of ssDNA-SH/Au electrode has been found to increases with increased scan rate (Fig. 4B), indicating slow electron-transfer kinetics.

3.6. Electrochemical response studies

The response studies have been carried out using cyclic voltammograms at scan rate of 20 Vs⁻¹ in PBS containing 40 µM MB (Fig. 5). The ssDNA/Au bioelectrode has been hybridized with different concentrations (100-500 ng) of genomic DNA for 5 minutes at room temperature (25°C). It has been observed that the magnitude of anodic peak current of MB increases with increase in the concentration of double stranded DNA (genomic DNA). The inset in Fig. 5 shows the curve between the magnitudes of peak current increases with concentration of genomic DNA. It appears that positively charged MB molecules are electrostatically attached to negatively charged phosphate backbone of double stranded DNA or get intercalated after hybridization. Moreover, the observed enhanced MB peak current with increased concentration of genomic DNA due to presence of more free guanine bases (unhybridized bases) of genomic DNA indicates increased interaction of MB with bases resulting in increased peak current [38]. It has been observed that increase in the MB peak with respect to genomic DNA concentration follows Eq. (1).

\[ I_{hybridized \ DNA} = 96.8 \times 10^{-2} + 6.9 \times 10^{-3} \times [genomic \ DNA \ conc.] \] \quad \text{(Eq.1)}

The specificity of the ssDNA/Au electrode has been monitored with the non-complementary DNA, resulting in no change in current as compared to that of ssDNA probe, indicating that no

\[ \text{Figure 5.} \text{ Response studies of ssDNA-SH/Au after hybridization with different concentration of genomic DNA (100-500) at 20 Vs}^{-1} \text{ in PBS (50 Mm, pH 7 containing 0.9 % NaCl) in 40 µM MB and inset shows the linear graph between current and concentration of genomic DNA.} \]
hybridization occurs. The hybridization time of probe with genomic DNA has been obtained as about 5 min. The sensitivity of dsDNA/Au electrode has been obtained as 0.027µA/ng cm⁻² with 0.978 regression coefficients (R). The ssDNA/Au electrode can detect *Vibrio cholerae* in the range of 10-60 ng/µl. The stability of ssDNA/Au electrode is obtained to be about 4 months when stored at 4°C. Table 1 shows characteristics of the ssDNA-SH/Au bioelectrode for cholera detection along with those reported in the literature.

**Table 1.** The characteristics of ssDNA-SH/Au bioelectrode for cholera detection along with those reported in the literature.

| Sl. No. | Surface/matrix | Transducer | Sensitivity | Detection limit | Linearity | Reference |
|---------|----------------|------------|-------------|----------------|-----------|-----------|
| 1       | Gold transducer surface | Quartz crystal microbalance | - - - - - - - - | 10⁵ cells/ml | 10 MHz AT | [39]       |
| 2       | Gold-coated AFM microcantilevers | Microcantilever-based biosensor | 146.5 pg/Hz | 1 × 10³ CFU/ml | 1 × 10⁻³ to 1 × 10⁷ CFU/ml | [40]       |
| 3       | Polytyramine-modified gold electrode | Immunosensor | 9×10⁻²⁰ M or 0.09 aM | 9×10⁻²⁰ M or 0.09 aM | 0.1 aM and 100 pM | [41]       |
| 4       | Au electrode | DNA biosensor | 0.027µA/ng cm⁻² | 100 ng | 100-500 ng | Present Work |

4. Conclusions
A self-assembled monolayer based electrochemical DNA biosensor has been fabricated by immobilizing thiolated probe (ssDNA-SH/Au) onto gold electrode surface and it’s hybridized with genomic DNA (dsDNA/Au) for detection of *Vibrio cholerae*. It has been concluded that ssDNA-SH/Au exhibits higher sensitivity (0.027µA/ng cm⁻²) with regression coefficient as 0.978 (R). The hybridization time of probe with genomic (dsDNA) at Au electrodes is 5 min with response time 60s. This electrochemical DNA sensor is highly specific for the detection of *Vibrio cholerae* in clinical samples. Efforts are being made to utilize this DNA probe for diagnosis of Cholera directly using patient’s samples.

Acknowledgements
Authors thank Director, NPL, New Delhi, India for the facilities. MKP is grateful to the Council of Scientific and Industrial Research (CSIR), India for the award of Senior Research Fellowship (SRF). Authors are thankful to Md. Azahar Ali for suggestion and support and thankful to Mr. Sandeep Singh for AFM measurements.

References
[1] Alam M, Hasan N A, Sultana M, Balakrish G, Sadique N A, Faruque A. S. G, Endtz H P, Sack RB, Huq A, Colwell R R, Izumiya H, Morita M, Watanabe H and Cravioto A 2010 *J. Clin.Microbiol.* **48** 3918-3922
[2] Faruque S M, Albert M J and Mekalanos J J 1998 *Microbiol. Molecul. Biol. Rev.* **68** 1301-1314
[3] Kelly P 2011 *Medicine* **39** 201-206
[4] Fournier J M and Quilici M L 2007 *Presse Medicaile* 36 727-739
[5] Louis V, Estelle R, Choopun N, Rivera I N G, Gangle B, Jiang S C, Rubin A, Patz J A, Hug A and Colwell R R 2003 *Appl. Environmen. Microbiol.* 69 2773-2785
[6] Gubala A J 2006 *J. Microbiological Meth.* 65 278-293
[7] Jyoung J Y, Hong S, Lee W and Choi J W 2006 *Biosens. Bioelectron.* 21 2315-2319
[8] Nikoleli G P, Nikolelis D P and Tzamtzisa N 2011 *Electroanal.* 23 2182-2187
[9] Martinez G A, Ambrosio J, Gutierrez C L and Flisser A 2001 *Clinic. Diagno. Lab. Immunol.* 28 768-771
[10] Rivera I N G, Lipp E K, Gil A, Choopun N, Huq A and Colwell R R 2003 *Environ. Microbiol.*
[11] Ligler F S, Taitt C R, Shriver L L C, Sapsford K E, Shubin Y and Golden J P 2003 *Anal. Bioanal. Chem.* 377 469-477
[12] Yamazaki W, Seto K, Taguchi M, Ishibashi M and Inoue K 2008 *BMC Microbiology* 8 94
[13] Belluzo M S, Ribone M E and Lagier C M 2008 *Sensors* 8 1366-1399
[14] Ciminska M G 2006 *Microbial Cell Fact.* 5 9
[15] Patel M K, Solanki P R, Kumar A, Khare S, Gupta S and Malhotra B D 2010 *Biosens. Bioelectron.* 25 2586-2591
[16] Souteyrand E, Cloarec J P, Martin J R, Wilson C, Lawrence I, Michelson S and Lawrence M F 1997 *J. Phys. Chem. B.* 101 2980-2985
[17] Berney H, West J, Haefele E, Alderman J, Lane W and Collins J K 2000 *Sens. Actua. B.* 68 100-108
[18] Arora K, Chand S and Malhotra B D 2006 *Anal. Chim. Acta* 568 259-274
[19] Wei F, Lillehoj P B and Ho C M 2010 *Pediat Res.* 67 458-468
[20] Matharu Z, Solanki P R, Gupta V and Malhotra B D 2012 *Analyst* 137 747-753
[21] Arya S K, Solanki P R, Datta M and Malhotra B D 2009 *Biosens.Bioelectron.* 24 2810-2817
[22] Gooding J J, Mearns F, Yang W and Liu J 2003 *Electroanal.* 15 81-96
[23] Cabrita J F, Abrantes L M and Viana A S 2005 *Electrochim. Acta* 50 2117-2124
[24] Gooding J J, Pugliano L, Hibbert D B and Erokhin P 2000 *Electrochem. Commun.* 2 217-221
[25] Li Y, Huang J, Mciver R T and Hemminger J C 1992 *J. Am. Chem. Soc.* 114 2428-2432
[26] Kim D C and Kang D J 2008 *Sensors* 8 6605-664
[27] Zhao Y D, Pang D W, Hu S, Wang Z L, Cheng J K and Dai H P 1999 *Talanta* 49 751-756
[28] Subramanian A, Irudayaraj J and Ryan T 2006 *Biosens. Bioelectron.* 21998-1006
[29] Oh B K, Kim Y K, Park KW, Lee W H and Choi J W 2004 *Biosens. Bioelectron.* 19 1497-1504
[30] Su X L and Li Y 2004 *Biosens. Bioelectron.* 19 563-574
[31] Prabhakar N, Arora K, Arya S K, Solanki P R, Iwamoto M, Singh H and Malhotra B D 2008 *Analyst* 133 1587-1592.
[32] Lin X, Wu P, Chena W, Zhang Y and Xia X 2007 *Talanta* 72 468-471
[33] Marrazza G, Chiti G, Mascini M and Anichini M 2000 *Clin. Chem.* 46 31-37
[34] Arora K, Prabhakar N, Chand S and Malhotra B D 2007 *Anal. Chem.* 79 6152-6158
[35] Patel M K, Solanki P R, Seth S, Gupta S, Khare S, Kumar A and Malhotra B D 2009 *Electrochem. Commun.* 11 969-973
[36] Yau H C, Chan H L and Yang M 2003 *Biosens. Bioelectron.* 18 873-879
[37] Gao H, Qi X, Chen Y and Sun W 2011 *Anal. Chim. Act.* 704 733-738
[38] Solanki P R, Patel M K, Kaushik A, Pandey M K, Kotnala R K and Malhotra B D 2011 *Electroanal.* 23 2699-2708
[39] Chen H, Zheng Y, Jiang J H, Wu H L, Shen G L and Yu R Q 2008 *Biosens. Bioelectron.* 24 684-689
[40] Sungkanak U, Sappat A, Wisitsoraat A, Promptmasa C, Tuantranont A 2010 *Biosens. Bioelectron.* 26 784-789.
[41] Loyprasart S, Hedstroma M, Thavarungkul P, Kanatharana P, Mattiasson 2010 *Biosens. Bioelectron.* 25 1977-1983.