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speB Genosensor for Rapid Detection of *Streptococcus pyogenes* Causing Damage of Heart Valves in Human

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Abstract

speB Genosensor for early detection of *Streptococcus pyogenes* in human throat swab samples were fabricated using screen printed modified gold electrode. The electrochemical changes before and after hybridization was measured using cyclic voltammetry (CV) in the presence of redox indicator. The **s**ensitivity of the genosensor was 80(μA/cm^2)/ng and lower limit of detection (LOD) was 0.10 ng/6μl with the regression coefficient (R^2) of 0.921 using CV. The sensor is highly specific, simple and takes only 30 min for detection of *S. pyogenes* infection. The sensor was further characterized by atomic force microscopy (AFM). Genosensor was found stable upto 6 months with only 10% loss in initial peak current in CV analysis on storage at 4°C. The *speB* gene based sensor can be used to prevent the damage of mitral and aortic heart valves by early diagnosis and treatment of the disease.

Keywords: Genosensor; Rheumatic heart disease; *speB* sensor; *Streptococcus pyogenes*

Abbreviations: AFM: Atomic Force Microscopy; CV: Cyclic Voltammetry; DPV: Differential Pulse Voltammetry; EI: Electrochemical Impedance; LOD: Limit of Detection; RHD: Rheumatic Heart Disease; SPGE: Screen Printed Gold Electrode; MB: Methylene Blue; LOD: Limit of Detection

Introduction

Streptococcus pyogenes infection in human throat causes initially pharyngitis and if it is not treated leads to damage of mitral and aortic heart valves a condition called rheumatic heart disease (RHD) [1-9]. The most common symptoms of rheumatic infection are joint pain, fever, chest pain or palpitations caused by heart inflammation (carditis), a rash and small bumps (nodules) under the skin [4,5]. The adherence of bacteria to the host cells as the initial event in the pathogenic process is a potential target for anti-adherence therapy, in which analogues of receptor molecule are used to prevent the bacteria from binding to the host cells [8,9]. The patient remains infected for weeks even after symptomatic resolution of pharyngitis and may serve as reservoir of infection to others. Most of the current tests rely on laboratory methods in addition to clinical symptoms, clinical history and geographic location of the patient which are not useful for therapy and prognosis of the disease [10-14].

Recent developments in new diagnostic tools, however, have opened doors for diagnosis of rheumatic heart infection. A number of serology based highly specific and sensitive assays such as falcon assay screening test ELISA [7], Dot- ELISA [13,14], rapid antigen detection system [15-20] and luciferase immunoprecipitation system [3] have been emerged. Molecular based approaches such as loop mediated isothermal amplification [15], RT-PCR [12] and PCR [10,11] are potential approach in the diagnosis of the disease. However, these tests are time-consuming, expensive and non-confirmatory based on single test. Now a days, nucleic acid based biosensors are gaining more attention over traditional diagnostic methods as they are faster, simpler, economical and more sensitive [1,21].

Electrochemical DNA biosensors are based on covalent attachment of DNA probe onto the surface of working electrode. In this method athiol labeled probe can be immobilized onto the gold electrode surface with strong covalent bonding [16-22] and the electrochemical changes after hybridization can be measured by cyclic voltammetry (CV) using methylene blue as redox indicator [6,19]. The present study reports the immobilization of 5'-thiol labeled *speB* gene specific DNA probe onto screen printed gold electrode surface for detection *S. pyogenes* causing damage of the heart valves.

Materials and Methods

Sample collection and chemicals

The gram positive *S. pyogenes* culture was taken from Institute of Microbial Technology (IMTECH), Chandigarh, India and the patient's throat swab samples were collected from Lady Harding Medical College, Delhi and Safdarjung Hospital, Delhi. Chloroform, EDTA, isoamyl alcohol, phenoland sodium chloridewere from Qualigens, India. Lysozyme, methylene blue (MB), RNase and Tris (Trizma base) were purchased from Sigma-Aldrich, USA. DNA purification kit (GFX column) was purchased from Amersham Biosciences Ltd, UK. Brain heart infusion broth was purchased from Himedia. 5′-thiol modified *speB* gene specific single stranded DNA probe (5′-GTAGCAACACATCCTGTAGCTGCA-3′) was procured from Bio India Life Sciences, India. All other chemicals were of analytical grade and purchased from local distributors in India. Screen-printed gold electrode (SPGE) was procured from DropSens, Spain and modified at Institute of Genomics and Integrative Biology (IGIB), India.

Isolation of genomic DNA

The genomic DNA (G-DNA) was isolated from 18 h Brain

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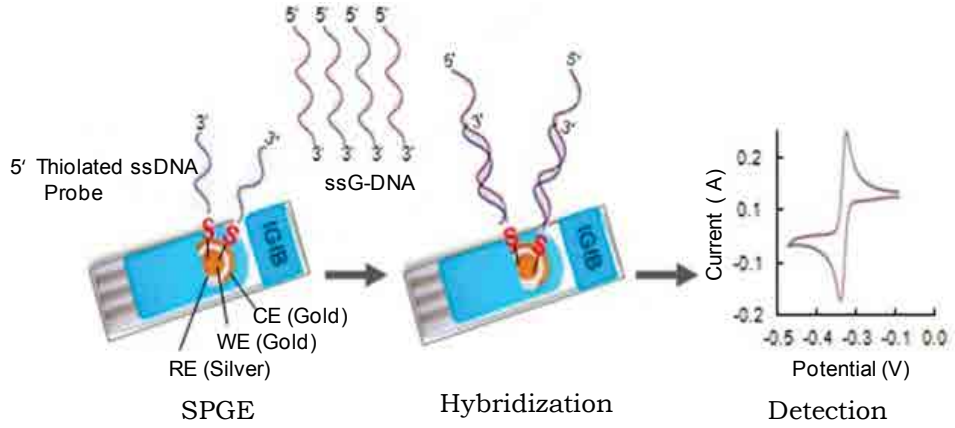

Scheme 1: Schematic representation of immobilization of 5´-thiol labeled speB ssDNA probe on SPGE and hybridization with ssG-DNA of S. pyogenes.

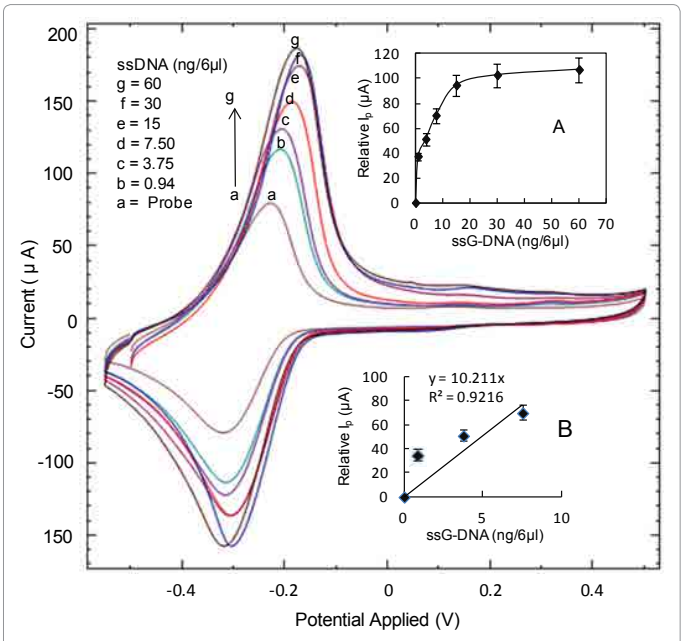

Figure 1: CV of (a) ssDNA-SH/Au and (b-g) hybridization with 0.94, 3.75, 7.50, 15, 30 and 60 ng/6µl of S. pyogenes ssG-DNA at 50 mVs-1 using 1 mM MB in 50 mM PBS, pH 7. The inset-A shows hyperbolic curve from 0-60 ng/6µl with linear peak current (Ip) up to 7.5 ng/6µl of ssG-DNA of S. pyogenes. Inset-B shows the linear plot from 0-7.5 ng/6µl ssG-DNA for the calculation of sensitivity and LOD.

heart infusion broth culture (grown at 37°C) [19]. The purity ($A_{260/280}$) and quantity of DNA (A_{260}) was measured using Nanodrop spectrophotometer. The G-DNA was also isolated from suspected patient throat swab (infected with *S. pyogenes*) samples. The swab was suspended in 100 µl TE buffer (10 mM Tris and 1mM EDTA), pH 8 and heated at 95°C for 5 min for bacterial lysis. After this, it was centrifuged at 5,000 × g for 2 min and supernatant was collected for quantification of DNA. The isolated double stranded genomic DNA solution (dsG-DNA) was denatured at 95°C to make single stranded genomic DNA (ssG-DNA) and used directly for hybridization with immobilized probe on electrode surface.

Construction of genosensor

The screen printed gold electrode (working: gold, counter: gold and reference: silver) with working surfacearea (0.126 cm^2) was cleaned with piranha solution (3:1, v/v concentrated sulphuric acid and 30% hydrogen peroxide solution) for 5 min at 25°C. The electrode was further washed with 70% ethanol and TE buffer, pH 8, respectively and dried at 25°C (room temperature). The thiolated 10 µM DNA probe (6 µL) was immobilized onto screen printed working gold electrode surface and kept for 24h at 25°C. The unbound probe was removed by repeated washings with TE buffer, pH 8. The different concentrations of ssG-DNA (0.94-60 ng/6µl) after denaturation at 95°C for 5min were hybridized in the presence of TE buffer, pH 8 for 10 min withprobe immobilized onto gold working electrode surface (0.126 cm^2). The electrochemical changes after hybridization were studied in the presence of redox indicator 1 mM methylene blue (MB) in PBS (50 mM sodium phosphate Buffer, 0.9% NaCl), pH 7 using cyclic voltammetry (CV)(FRA2 µAutolab type iii, Metrohm, India). After hybridization, the electrode surface was washed 3-4 times with TE buffer, pH 8 followed by PBS, pH 7 to remove the unbound ssG-DNA. The fabrication of the genosensor is shown in Scheme 1.

Characterization of sensor

The surface topography of bare gold electrode, thiol labeled ssDNA probe and hybridized DNA (dsDNA) was delineated using Atomic Force Microscopy (5500 scanning probe microscope, Agilent Tech., USA) in a non-contact mode.

Results and Discussion

CV Studies

CV of immobilized singlestranded DNA probe and after hybridization with different concentrations of ssG-DNA is shown in Figure 1. The increase in oxidation peak current along with an increase in ssG-DNA was due to the presence of extra unhybridized bases of G-DNA that interacted with MB molecules. The ssDNA-SH/Au electrode consists of the limited number of unhybridized nitrogenous bases that interact with MB, while ssG-DNA comprises of prolonged unhybridized ssDNA sequences consisting of uncountable nitrogenous bases, resulting an increased interaction with MB molecules. This leads to increase oxidation peak current in dsDNA/Au electrode as compared to ssDNA-SH/Au electrode.

The graph of relative peak current (I_p) with respect to probe (as zero) and ssG-DNA concentrations is hyperbolic and showed the increase in I_p up to 40 ng/6µl ssG-DNA (Figure 1, inset A). On further increasing the concentration of hybridizing ssG-DNA, the I_p does not

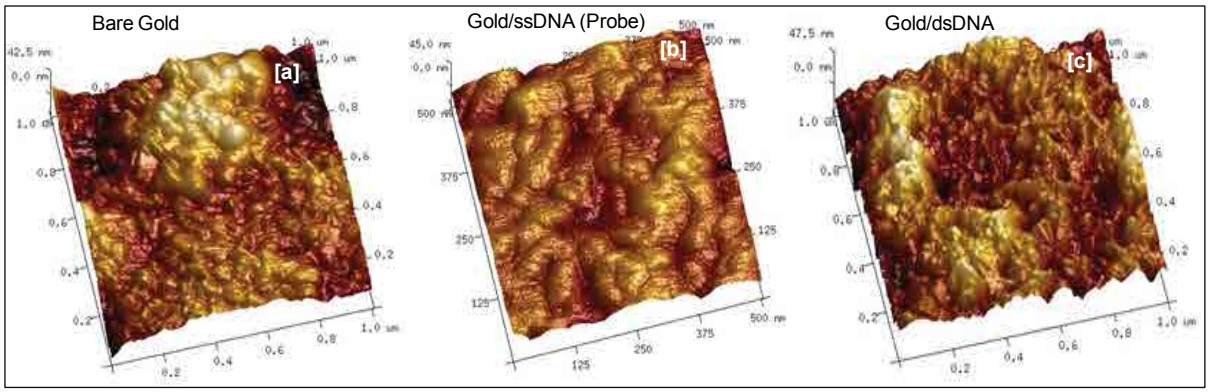

Figure 2: AFM micrograph of (a) bare gold electrode (b) gold/ssDNA probe and (c) gold/dsDNA after hybridization with 60 ng/6 µl ssG-DNA of S. pyogenes.

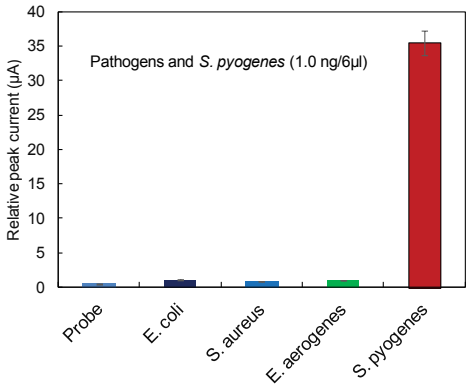

Figure 3: Specificity test of speB genosensor for S. pyogenes and other possible pathogens. The relative Ip value of CV (with respect to immobilized probe as zero) after hybridization with 1.0 ng/6µl ssG-DNA of other pathogens and S. pyogenes.

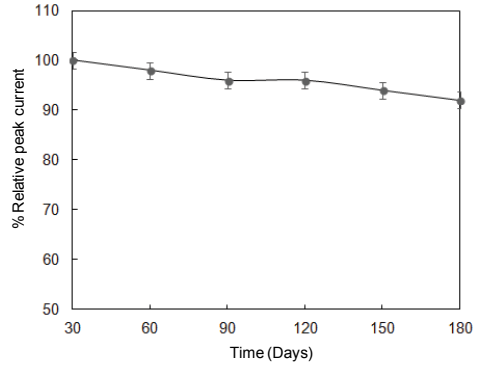

Figure 4: Stability of the sensor measured as % relative peak current(with respect to probe as zero using CV as described above) with a regular interval of 30 days for 6 months on storage at 4oC. Each value is the average of three readings under same conditions.

increase further as all immobilized ssDNA probe on working electrode surface get saturated at 40 ngssG-DNA and is not available for further hybridization with ssG-DNA(>40 ng). For calculation of sensitivity and LOD the concentrations of probe as zero (0), 3.75 and 7.50 ng/6 µl ssGDNA of *S. pyogenes* were used to achieve best regression coefficient. The plot from 0 -7.5ng/6µl ssG-DNA showed the linear equation I_p (µA) = 10.21 (µA/ng) × ssG-DNA(ng) +0 (intercept) with regression coefficient (R^2) 0.921 (Figure 1, inset B). The Sensitivity (S) of the genosensor was 80 (µA/cm^2)/ng which was calculated using the formula; S = m/A here, m is the slope of the linear equation and A is the area of the working gold (0.126 cm^2) electrode. The limit of detection (LOD) was found approximately 0.10 ng/6µl using the formula LOD = 3(σ/S) where σ is the standard deviation and S is the sensitivity.

Characterization of the sensor (Atomic force microscopy)

The morphological changes on gold working area were studied using atomic force microscopy for confirmation of the immobilization and hybridization of probe with bacterial DNA samples. The changes in surface roughness of bare gold electrode, after immobilization of ssDNAprobe and after hybridization of ssG-DNA with probe(dsDNA) are shown in Figure 2. AFM image of bare gold electrode showed a smooth surface area with average roughness (R_a) of 4.39 nm (Figure 2a). After immobilization of thiol labeled *speB* gene based probe surface roughness increased to 8.30 nm (Figure 2b) and further, after hybridization with ssG-DNA of *S. pyogenes,* the surface roughness increased to 11.2 nm (Figure 2c). The change in surface morphology of gold working electrode and increase in roughness R_a confirms the immobilization of thiol labeled probe and hybridization with ssG-DNA of *S. pyogenes* [2].

Specificity and stability of the sensor

The specificity of the sensor was checked with *S. pyogenes* and other possible pathogens found in suspected patient throat swab samples, after hybridization with immobilized *speB*genes specific ssDNAprobe on gold screen printed working electrode surface. The relative peak current (I_p) after hybridization of ssDNA probe with 1.0ng/6µl of ssG-DNA of *Escherichia coli, Staphylococcus aureus, Enterobacter aerogenes* and *S. pyogenes* respectivelyare shown in Figure 3. The relative I_p (with respect to probe as zero) of the genosensor with other pathogens (*E. coli, S. aureus and E. aerogenes*) were found almost the same with immobilized ssDNA probe (no change in current) except *S. pyogenes* which confirms the specificity of the sensor only to *S. pyogenes***.** The stability of the genosensor (gold electrode with immobilized probe) on storage at 4°C was studied using CV at regular interval of 30 days (Figure 4). The sensor was found stable up to 6months at 4°C with approximately 10% loss in original CV current.

Conclusion

The sensitivity of the genosensor developed in the present study for the detection of *S. pyogenes*was 80(µA/cm^2)/ng and lower limit of detection (LOD) was 0.10 ng/6µl with the regression coefficient (R^2)

0.921 using CV. The *speB* genosensor can detect approximately up to 0.10 ngss G-DNA/6µl directly from swab samples of patient infected with *S. pyogenes* without culturing and isolating G-DNA in 30 min. There, the developed genosensor is highly specific to *S. pyogenes* and can detect infection only in 30 min to prevent damage of mitral and aortic heart valves.

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