Amperometric Detection of Pathogen Causing Rheumatic Heart Disease

Singh S1,2, Kaushal A1, Gupta S3 and Kumar A1,2*
1CSIR-Institute of Genomics and Integrative Biology, Mall Road, Delhi-110007, India
2Academy of Scientific and Innovative Research (AcSIR), New Delhi, 110025, India
3National Centre for Disease Control, Sham Nath Marg, Delhi-110054, India

Abstract

Rheumatic heart disease (RHD) is the damage of mitral or aortic heart valves due to delayed treatment of rheumatic fever. Streptococcus pyogenes, representative of Group A Streptococcus is the causative agent of the disease. The current diagnostic methods are either have some limitations or non-confirmatory due to single test. Hence, nucleotide based sensors may serve as reliable and rapid method for the diagnosis of the RHD disease. A 5’carboxyl labeled single stranded DNA probe complementary to mga gene of pathogen Streptococcus pyogenes was immobilized on screen printed gold electrode modified with mercaptopropionic acid and poly (amidoamine) dendrimer. Genomic DNA of pathogen was isolated from throat swab of suspected RHD patients and denatured at 94°C to make single strand DNA for hybridization with immobilized probe using redox indicator and electrochemical response was measured amperometrically using cyclic voltammetry. The sensitivity of the sensor was found 743 (µA/cm²)/ng and limit of detection was 0.18 pg/6 µL with regression coefficient (R²) 0.9622. The sensor was found highly specific to S. pyogenes and stable upto six months on storage at 4°C with only 10% loss in initial activity.

Keywords: Amperometric; mga sensor; Pathogen; Rheumatic heart disease; Streptococcus pyogenes

Abbreviations: CV: Cyclic Voltammetry; FISH: Fluorescence In-situ Hybridization; LOD: Limit of Detection; MPA: Mercaptopropionic Acid; PAMAM: Poly (Amidoamine); RADT: Rapid Antigen Detection Test; RHD: Rheumatic Heart Disease

Introduction

Pathogen Streptococcus pyogenes infects throat of humans and causes pharyngitis at initial stage and at later stages may lead rheumatic fever which may damage mitral and aortic heart valves called rheumatic heart disease (RHD) [1]. In humans, Group A Streptococcus causes wide range of infections. Manifestations of disease can result in pneumonia, bacteremia, necrotizing fasciitis, upper respiratory tract infections, skin and soft tissue infections and streptococcal toxic shock syndrome. In developing countries RHD is the main cause of cardiac disease. Mostly, 65% to 70% patients suffers with damage of mitral valve whereas, 25% patients with aortic valve [2,3]. Every year, about 2.3 lakh people die due to RHD [2,4-6]. Socio-economic and environmental factors like overcrowding, poverty and malnutrition play a bigger role in spreading of rheumatic fever [7,8]. Traditional diagnostic techniques for detection of bacterial pathogen S. pyogenes infection include bacterial culture test, rapid antigen detection test (RADT), fluorescence in-situ hybridization (FISH), biochemical test, serological test, C-reactive protein (CRP) test, ESR, PCR, bacitracin susceptibility, phadebact test and genetic markers [9-11]. Most of these tests show high specificity but low sensitivity. The present diagnostic methods are time consuming, expensive, nonspecific, less sensitive and suffer many limitations as well as non-confirmatory on single test.

The early diagnosis of RHD is very important for saving patient life in time. Therefore, a sensitive and fast technology is required for early detection of RHD. The biosensor technique is very sensitive, quick and accurate method which can help in early diagnosis of the disease for better care for health [12].

Screen-printing electrodes are currently used for lab-on-chip based sensor for diagnosis of diseases due to rapid, disposable and suitable for point-of-care diagnosis of infectious diseases [13]. In electrochemical DNA sensors immobilized probe is used as recognition element that binds to specific target molecule. The main advantages of electrochemical biosensors are their sensitivity, selectivity and reliability [14,15]. Here, we report immobilization of specific DNA probe based on mga gene of S. pyogenes onto gold modified nanohybrid electrode surface for detection of pathogenic bacteria S. pyogenes causing damage of human heart valves.

Materials and Methods

Sample collection and chemicals

The patient’s throat swab samples were collected from ENT Department, Safdarjung Hospital, Delhi. Chloroform, EDTA, isoamyl alcohol, phenol, sodium chloride, sodium di-hydrogen ortho-phosphate and di-sodium hydrogen orthophosphate were obtained from Qualigen, India. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-Hydroxysuccinimide (NHS), lysozyme, mercaptopropionic acid (MPA), methylene blue (MB), poly amidoamine (PAMAM) dendrimer 3rd generation, RNase and Tris (Trizma base) were purchased from Sigma-Aldrich, USA. Brain heart infusion broth media was purchased from Himedia, India. 5’ carboxyl modified mga gene specific single stranded DNA (ssDNA) probe (5’ HOOC-GCACAGCCAATTTCTAGCTTGTCG 3’) was procured from Bio India Life Sciences, India. All other chemicals were analytical grade and purchased from local suppliers in India. Screen-printed gold electrode (SPGE), consist of three electrode system (gold (Au) as working and counter and silver (Ag) as reference electrode) was procured from DropSens, Spain and modified in our lab for development of nanohybrid sensor.

*Corresponding author: Kumar A, CSIR-Institute of Genomics and Integrative Biology, Mall Road, Delhi-110007, India, Tel: 011 2987 9487; E-mail: ashokigb@rediffmail.com

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Isolation and hybridization of genomic DNA

The genomic DNA (G-DNA) was isolated from patient’s throat swab samples as described earlier [10, 16]. The purity of the DNA sample was determined by 260/280 ratio (~1.8 for pure DNA samples) using Nanodrop spectrophotometer. The different concentrations of patients G-DNA solutions (dsDNA) were denatured at 95°C for 5 min to make single stranded DNA (ssG-DNA) to hybridize with immobilized ssDNA probe on modified composite nanohybrid electrode (Au/MPA/PAMAM) for 10 min.

Construction of the sensor

A screen printed electrode with gold as working (0.126 cm²) washed sufficiently with water and dried. After drying, 6 µL MPA (99%) was placed overnight onto the working surface of the electrode which binds with –SH groups to gold and form monolayer of MPA. An unbound MPA was removed thoroughly by several washing with Milli-Q water and dried at room temperature (25°C). The working electrode was further treated with (6 µL) equimolar mixture of 10 mM EDC and NHS (1:1, v/v) in Milli-Q water for 1h to activate the carboxyl groups on the surface and washed electrode with water and dried at room temperature. Further, 6 µL PAMAM (165 ng/µL in water) was placed on electrode and incubated for 2 h to form amide bond by binding few amino groups of PAMAM to carboxyl groups of MPA through EDC-NHS chemistry [10]. After incubation, the electrode was washed 3–4 times with water to remove unbound materials and dried at room temperature.

Further, a mixture of 3 µL (10 µM 5’carboxyl modified ssDNA probe) and 3 µL (10 mM EDC and NHS [1:1, v/v]) was placed on electrode for 3 h for formation of amide bond between the –COOH group of the probe and –NH₂ group of the PAMAM to make Au/MPA/PAMAM/ssDNAprobe on working electrode [10]. The unbound probe and other chemicals were removed by several washing with Milli-Q water and further washed with TE (10 mM Tris, 1 mM EDTA) buffer, pH 8.0 and dried at room temperature. The ssG-DNA different concentrations (0.01-10 ng/6 µL) were made by denaturation at 95°C for 5 min in TE buffer, pH 8.0 which was hybridized onto the Au/MPA/PAMAM/ssDNAprobe for 10 min at room temperature. After hybridization the electrode was washed with water and then 3–4 times with PBS (50 mM sodium phosphate buffer, 0.9% sodium chloride), pH 7.0 and dried as usual. Electrochemical measurements were taken by cyclic voltammetry (FRA2 µAutolab type iii, Metrohm, India) using redox indicator 50 µL methylene blue (5 mM MB in PBS, pH 7.0) [17].

Results and Discussion

Amperometric studies using cyclic voltammetry (CV)

The CV (Figure 1) of Au/MPA/PAMAM/ssDNAprobe, peak current (I_p) was higher (29.31 µA) than that of bare Au (not shown) due to the increased binding of redox indicator MB molecules to ssDNA probe. The I_p of Au/MPA/PAMAM/dsDNA (after hybridization) was higher than that of Au/MPA/PAMAM/ssDNAprobe and it increases with the increase in concentrations of hybridizing ssG-DNA of bacterial pathogen S. pyogenes. The increase in peak current was observed due to more binding of MB on dsDNA as compared to ssDNA resulting in more oxidation of MB molecules on the surface of electrode and hence increased in the current. The plot between the ssDNA concentrations and relative Ip values with respect to probe (zero) was hyperbolic (Figure 1 inset [a]). It was linear for 0-0.1 ng/6 µL ssG-DNA following

The fabrication of amperometric DNA sensor for detection of pathogen S. pyogenes is shown in Scheme 1.
the linear equation \[ I_p (\mu A) = 93.619 (\mu A/ng) \times ssG-DNA (ng) + 0 \] and regression coefficient \( R^2 \) 0.9622 (Figure 1 inset [b]). The sensitivity (S) of the present DNA sensor was found 743 (µA/cm²)/ng which was calculated using the formula \( S = \frac{m}{A} \) where, \( m \) is the slope of the linear equation and \( A \) is the area of the working nanohybrid composite electrode (0.126 cm²). The limit of detection (LOD) of the sensor was 0.18 pg/6 µL (0.18 × 10⁻³ ng/6 µL) calculated using the formula LOD = \( \frac{3\sigma}{S} \) where, \( \sigma \) is the standard deviation and \( S \) is the sensitivity (Figure 1 inset [b]).

**Specificity and stability of the sensor**

The specificity of the Au/MPA/PAMAM/ssDNA探针 nanohybrid sensor with \( S. pyogenes \) human DNA and with other possible pathogens (\( E. coli \), \( E. aerogenes \), \( S. aureus \) and \( B. sphaericus \)) found in throat swab of the patients is shown in Figure 2. The relative \( I_p \) of the sensor after hybridization with \( ssG-DNA \) (1.0 ng/6 µL) with human genomic DNA and other pathogens found in throat swab were found in CV almost same as immobilized probe (zero) except with \( S. pyogenes \) which shows higher \( I_p \) even at lower concentration (0.01 ng/6 µL) of hybridization with ssG-DNA. The increase in relative \( I_p \) values was obtained only with \( S. pyogenes \), which confirms the specificity of the sensor to \( S. pyogenes \) no other pathogens.

The stability of the Au/MPA/PAMAM/ssDNA探针 composite nanohybrid electrode was measured by changing peak current (\( I_p \)) of CV at every month on storage at 4°C. The nanohybrid electrode was found stable on storage in cold for 6 months with only 10% loss in initial \( I_p \) value of CV (Figure 3).

**Conclusion**

The Au/MPA/PAMAM/ssDNA探针 composite nanohybrid DNA sensor can be used to detect pathogen at lower concentration (0.18 pg ssDNA in 6 µL sample) at early stage of bacterial infection (\( S. pyogenes \) doubles at every 30 min) to prevent damage of mitral and aortic heart valves (RHD). The sensor is highly specific to the DNA of \( S. pyogenes \) (due to \( mga \) gene specific probe) and can diagnose pathogen at early stage of infection only in 30 min to save damage of heart valves of patient by taking proper medical care.

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**References**