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# Amperometric Detection of Pathogen Causing Rheumatic Heart Disease

**Singh S[1,2], Kaushal A[1], Gupta S[3] and Kumar A[1,2]***

[1]*CSIR-Institute of Genomics and Integrative Biology, Mall Road, Delhi-110007, India*
[2]*Academy of Scientific and Innovative Research (AcSIR), New Delhi, 110025, India*
[3]*National 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/cm$^2$)/ng and limit of detection was 0.18 pg/6 µL with regression coefficient ($R^2$) 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, bacteraemia, 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 Qualigens, 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: ashokigib@rediffmail.com

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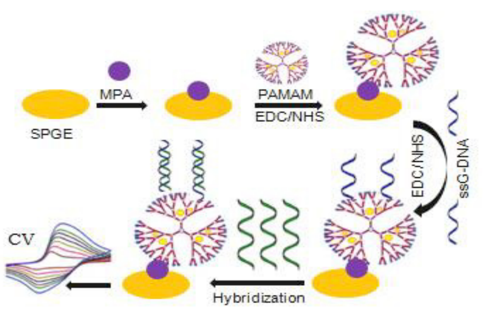
## Isolation and hybridization of genomic DNA

The genomic DNA (G-DNA) was isolated from patient's throat swab samples as descried 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 $cm^2$) 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 $-NH_2$ group of the PAMAM to make Au/MPA/PAMAM/$ssDNA_{probe}$ 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/$ssDNA_{probe}$ 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].




**Scheme 1:** Fabrication of Au/MPA/PAMAM/$ssDNA_{probe}$ composite nanohybrid DNA sensor for amperometric detection of pathogen causing RHD. [SPGE: Screen printed gold electrode, MPA: Mercaptopropionic acid, PAMAM: Poly(amidoamine), EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, NHS: *N*-Hydroxysuccinimide, ssDNA: Single stranded DNA, ssG-DNA: Single stranded genomic DNA, CV: Cyclic voltammetry].

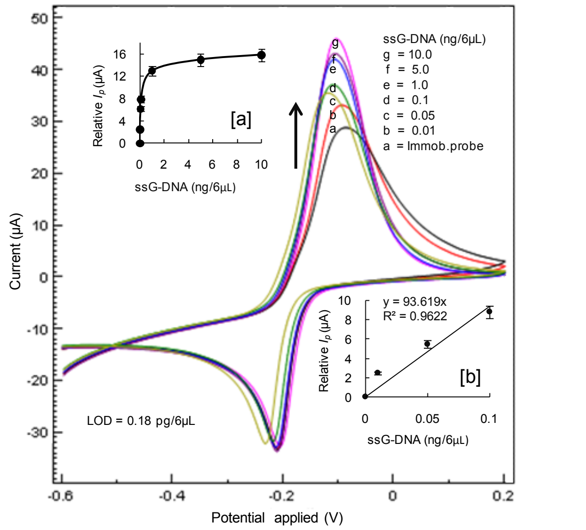



**Figure 1:** CV of (a) Au/MPA/PAMAM/$ssDNA_{probe}$ and (b-g) hybridization with 0.01, 0.05, 0.1, 1.0, 5.0 and 10 ng/6 µL ssG-DNA of *S. pyogenes* at 50 $mVs^{-1}$ using 5 mM MB in 50 mM PBS, pH 7. The inset [a] shows hyperbolic curve between relative *Ip* (with respect to probe as zero) with increasing concentrations of hybridizing ssG-DNA of *S. pyogenes* and inset [b] shows linear curve for detection of LOD.

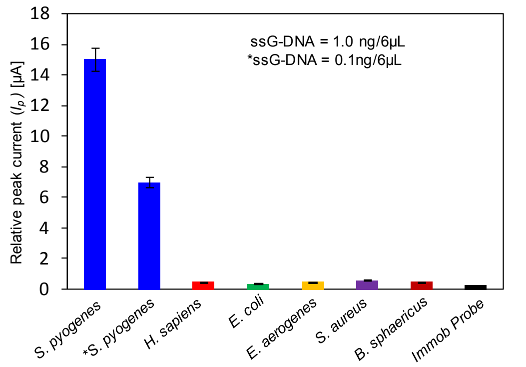



**Figure 2:** Specificity of the sensor for *S. pyogenes* and other possible pathogens. The relative $I_p$ value of CV (with respect to immobilized probe as zero) after hybridization with 1.0 ng/6 µl with human and other pathogens besides *S. pyogenes* (1.0 ng and 0.1 ng/6 µl).

The fabrication of amperometric DNA sensor for detection of pathogen *S. pyogenes* is shown in Scheme 1.

# Results and Discussion

## Amperometric studies using cyclic voltammetry (CV)

The CV (Figure 1) of Au/MPA/PAMAM/$ssDNA_{probe}$ 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/$ssDNA_{probe}$ and it increases with the increase in concentrations of hybridizing with 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

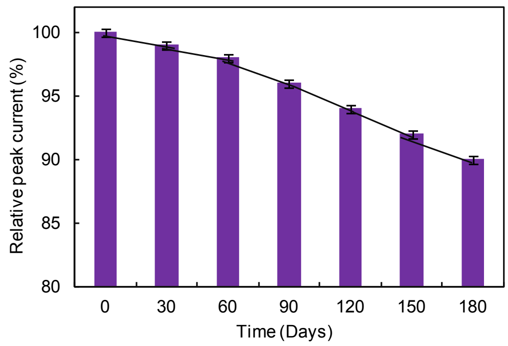



**Figure 3:** Stability of the sensor was measured as % relative peak current (with respect to probe as zero using CV as described above) at regular interval of 30 days on storage of the nanohybrid composite electrode at 4°C for 6 months. Each value is the average of three readings at same conditions.

the linear equation [$I_p$ (μA) = 93.619 (μA/ng) × 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/$cm^2$)/ng which was calculated using the formula S = m/A where, m is the slope of the linear equation and A is the area of the working nanohybrid composite electrode (0.126 $cm^2$). 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 = 3 (σ/S) where, σ 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/$ssDNA_{probe}$ 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/$ssDNA_{probe}$ composite nanohybrid electrode was studied by measuring change in 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/$ssDNA_{probe}$ 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

1. Kaushal A., Goyal L. & Kumar A.
Recent advances in the diagnosis of rheumatic heart disease.
Biosci. Biotech. Res. Comm. 2011, **4**: 1-9.

2. Guilherme L., Faé K., Oshiro S.E. & Kalil J.
Molecular pathogenesis of rheumatic fever and rheumatic heart disease.
Expert Rev. Mol. Med. 2005, **7**: 1-15.

3. Rajamannan N.M., Francesco A.C., Luis M., Jose L.Z., Raphael A.R. & Patricia J.M.
Medical therapy for rheumatic heart disease: Is it time to be proactive rather than reactive?
Indian Heart J. 2009, **61**(1): 14-23.

4. Carapetis J.R, McDonald M. & Wilson N.J.
Acute rheumatic fever.
Lancet 2005, **366**: 155-168.

5. Ellis N.M., Li. Y., Hildebrand W., Fischetti V.A. & Cunningham M.W.
T cell mimicry and epitope specificity of cross-reactive T cell clones from rheumatic heart disease.
J. Immun. 2005, **175**: 5448-5456.

6. Pedro M.A., Rosa R.P. & Luiza, G.
Understanding rheumatic fever.
Rheumatol. Int. 2012, **32**: 1113-1120.

7. Dajani A.S., Ayoub E.M., Bierman F.Z., Bisno A.L., & Deny F.W.
Guidelines for the diagnosis of rheumatic fever: Jones criteria.
Circulation 1993, **87**: 302-307.

8. Guilherme L., Köhler K.F., Postol E. & Kalil J.
Genes, autoimmunity and pathogenesis of rheumatic heart disease.
Ann. Pediatr. Cardiol. 2011, **4**(1): 13-21.

9. Kaushal A., Kumar D., Khare S. & Kumar A.
*speB* gene as a specific genetic marker for early detection of rheumatic heart disease in human. *Cell.* Mol. Biol. 2012, **58**(1): 50-54.

10. Singh S., Kaushal A., Khare S. & Kumar A.
Gold-mercaptopropionic acid-polyethylenimine composite based DNA sensor for early detection of rheumatic heart disease.
Analyst 2014, **139**: 3600-3606.

11. Uhl J.R. & Patel R.B.
Fifteen-minute detection of *Streptococcus pyogenes* in throat swabs by use of a commercially available point-of-care PCR assay.
J. Clin. Microbiol. 2016, **54**(3): 815-817.

12. Mascini M. & Tombelli S.
Biosensors for biomarkers in medical diagnostics.
Biomarkers 2008, **13**: 637-657.

13. Hui P., Lijuan Z., Christian S. & Jadranka T.S.
Conducting polymers for electrochemical DNA sensing.
Biomaterials 2009, **30**: 2132-2148.

14. Brahmadathan K.N. & Gladston P.
Microbiological diagnosis of Streptococcal pharyngitis: Lacunae and their implications.
Indian J. Med. Microbiol. 2006, **24**(2): 92-96.

15. Thévenot D.R., Toth K., Durst R.A. & Wilson G.S.
Electrochemical biosensors: recommended definitions and classification.
Biosen. Bioelectron. 2001, **16**(1): 121-131.

16. Singh S., Kaushal K., Khare S. & Kumar A.
*mga* Genosensor for early detection of human rheumatic heart disease.
Appl. Biochem. Biotechnol. 2014, **173**: 228-238.

17. Akiko T., Andrew J., Thomson B. & Julea N.
Methylene blue as an electrochemical discriminator of single and double-stranded oligonucleotides immobilised on gold substrates.
Analyst 2001, **126**: 1756-1759.