Gene Specific Impedimetric Bacterial DNA Sensor for Rheumatic Heart Disease

Swati Singh^{1,2} · Ankur Kaushal¹ · Sunil Gupta³ · Ashok Kumar^{1,2}

Received: 26 August 2016 / Accepted: 14 September 2016 / Published online: 22 September 2016
© Association of Microbiologists of India 2016

Abstract An impedimetric *mga* gene specific DNA sensor was developed by immobilization of single stranded DNA probe onto the screen printed modified gold-dendrimer nanohybrid composite electrode for early and rapid detection of *S. pyogenes* in human throat swab samples causing rheumatic heart disease. Electrochemical impedance response was measured after hybridization with bacterial single stranded genomic DNA (ssG-DNA) with probe. The sensor was found highly specific to *S. pyogenes* and can detect as low as 0.01 ng ssDNA in 6 μ L sample only in 30 min. The nanohybrid sensor was also tested with non-specific pathogens and characterized by FTIR. An early detection of the pathogen *S. pyogenes* in human can save damage of mitral and aortic heart valves (rheumatic heart disease) by proper medical care.

Keywords DNA sensor · Impedimetric · Nanohybrid · PAMAM · Rheumatic heart disease · *S. pyogenes*

Streptococcus pyogenes is a gram-positive extracellular bacterial pathogen. The bacteria colonize in the throat and are responsible for initial infection pharyngitis and if it is

not treated may leads to rheumatic fever [1–5]. Rheumatic heart disease (RHD), causing damage of heart valves, is the most serious autoimmune sequelae of streptococcal infection which leads to disability and death among children worldwide. The RHD may leads to chronic valvular lesions (mitral and aortic valves). The similar epitopes of bacteria and host (molecular mimicry), is the major cause of triggering the disease. Kalia and his groups published several papers on genome wide approach for identification of several unique biomarkers for identifying several species (strains) of *Streptococcus*, *Staphylococcus*, *Yersinia*, *Vibrio*, *Clostridium*, *Lactobacillus* and many other bacterial pathogens [6–12]. Current methods of detection of *S. pyogenes* infection are bacterial culture test, rapid antigen detection test (RADT), biochemical test, serological test, C-reactive protein (CRP) test, ESR, PCR, bacitracin susceptibility and Phadebact test [13–15]. All traditional diagnostic methods for detection of *S. pyogenes* are time consuming, expensive, non specific, less sensitive and suffer one or more limitations. DNA biosensors are the modern diagnostic technique which greatly increases the sensitivity and specificity of the detection of pathogens [16, 17]. DNA sensor is based on hybridization of genomic DNA of *S. pyogenes* with probe which is highly specific to *S. pyogenes* [13, 14].

Our aim is to develop a rapid, accurate, sensitive and cost effective technique for the detection of *S. pyogenes* in suspected RHD patient samples. The present method describes the use of gold-poly (amidoamine) dendrimer (PAMAM) nanohybrid modified gold electrode for the early and quick impedimetric detection of *S. pyogenes* infection from suspected patient samples. If the target single stranded genomic DNA (ssG-DNA) contains a same complementary sequences of the immobilized single stranded DNA probe (ssDNA), a hybrid duplex dsDNA

Electronic supplementary material The online version of this article (doi:10.1007/s12088-016-0620-6) contains supplementary material, which is available to authorized users.

✉ Ashok Kumar
ashokigib@rediffmail.com

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

(double stranded DNA) is formed at the electrode surface which is detected by increasing in R_{ct} (resistance charge transfer diameter) using redox indicator potassium ferricyanide $[K_3Fe(CN)_6]^{3-/4-}$ [16]. The screen printed electrodes can be used for fabrication of biosensors due to economical, portable and disposable after testing the infectious samples. Thiol (–SH) modified ssDNA probe can be covalently immobilized on the gold (Au) surface [18]. Similarly, mercaptopropionic acid (MPA) which also contains thiol is attached to the Au electrode surface to form stable S–Au bond which can be used for development of electrochemical impedimetric DNA sensor. Polyamidoamine (PAMAM, 3rd generation from Sigma) dendrimer can be used in development of biosensor due to high symmetry in geometry, stability, size and surface modification functionality. The electrode surface can be modified using dendrimers due to their biocompatibility and adequate number of amine functional groups. The NH_2 groups of the PAMAM can be attached to the –COOH groups of MPA through covalent and stable amide bond formation by 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) chemistry [14, 19].

Screen printed gold electrode (SPGE, DropSens) with gold as working and counter electrode and silver as reference electrode was chemically modified for the development of the nanohybrid DNA sensor. 6 μ L of MPA (99 %) was placed on the working Au electrode surface (0.12 cm^2) for 24 h to form stable monolayers. The MPA attached to the Au electrode by its –SH groups. The electrode was then thoroughly washed with autoclaved distilled water to remove excess MPA and then dried at 25 °C. The equimolar solution 6 μ L of 10 mM EDC and NHS (1:1 v/v) in autoclaved Milli-Q water was kept for 10 min on electrode to activate the carboxyl groups of MPA. Then, the electrode was washed with Milli-Q water and further allowed to react 6 μ L PAMAM solution (160 ng/ μ L) for 3 h. An amide bond formed between –COOH groups of MPA and – NH_2 groups of PAMAM through EDC/NHS chemistry. The electrode was then thoroughly washed 3–4 times with water to remove excess PAMAM and dried at 25 °C. The 5'-carboxyl modified 24 mer ssDNA probe (5'-HOOC-GCACAGCCAATTTCTAGCTTGTCG-3') of *mga* gene (10 μ M in autoclaved Milli-Q water) was diluted 1:1 (v/v) with equimolar solution of 10 mM EDC and NHS (in autoclaved Milli-Q water) to make the final probe concentration 5 μ M. Then, 6 μ L of probe/EDC-NHS mixture was placed on working electrode for 3 h where the –COOH groups of the probe and – NH_2 groups of the PAMAM forms amide bond (EDC-NHS chemistry). Finally, the unbound probe was removed by washing 3–4 times with autoclaved Milli-Q water and dried at room temperature before electrochemical impedance detection. The genomic DNA (G-DNA) of *S. pyogenes* was isolated from patient

throat samples as described earlier [14, 15] and denatured at 95 °C for 5 min to make ssG-DNA. The Au/MPA/PAMAM/ss-DNA probe was hybridized with 0.01–5 ng/6 μ L ssG-DNA in TE buffer (10 mM Tris and 1 mM EDTA), pH 8.0 for 10 min at 0.126 cm^2 surface area of the Au working electrode. After hybridization, the Au/MPA/PAMAM/ds-DNA electrode was washed 3–4 times with TE buffer, pH 8.0, followed by PBS (50 mM sodium phosphate buffer containing 0.9 % sodium chloride), pH 7.0 and dried at room temperature (25 °C) before electrochemical measurements. The schematic fabrication of Au/MPA/PAMAM, immobilization of ss-DNA probe and hybridization with ssG-DNA of *S. pyogenes* to form Au/MPA/PAMAM/ds-DNA is shown in Scheme 1. The nanohybrid DNA sensor was further used to detect *S. pyogenes* electrochemically (FRA2 μ Autolab type iii, Metrohm, India) by measuring changes in electrochemical impedance (EI).

The Nyquist plot for impedance studies of Au/MPA/PAMAM/ss-DNA and Au/MPA/PAMAM/dsDNA is shown in Fig. 1. The charge transfer resistance (R_{ct} , Ohm) is considered equal to the diameter of the semicircle interface of the electrode [16, 20]. The Nyquist complex impedance obtained, best fit to Randles equivalent circuit of electrochemical interface where, R_s corresponds to the electrolyte resistance, C_{dl} for double layer capacitance, R_{ct} represents the charge transfer resistance and W_d is the Warburg diffusion element (Fig. 1 inset). C_{dl} is in series with R_s and parallel to R_{ct} [21]. The R_{ct} of bare Au was lower than that of Au/MPA/PAMAM (not shown in the figure). This might be due to the presence of dendrimer PAMAM in the nanohybrid which decreased the conductivity and hence increased the impedance. In case of Au/MPA/PAMAM/ssDNA complex the R_{ct} value was higher than Au/MPA/PAMAM. This may be due to the negatively charged phosphate groups of ssDNA which prevent the $[Fe(CN)_6]^{3-/4-}$ ions reaching from the surface of the electrode. The R_{ct} value of Au/MPA/PAMAM/dsDNA was higher than that of Au/MPA/PAMAM/ssDNA and it increases with increasing concentrations of hybridizing ssG-DNA and gets saturated at higher concentrations of *S. pyogenes* ssG-DNA (Fig. 1[B]). This may be due to more negatively charged phosphate groups on the electrode surface after hybridization which consequently increases the surface thickness and results an increase in the R_{ct} value which later gets saturated and further no increase in R_{ct} value. The limit of detection (LOD) of the sensor was found experimentally 0.01 ng/6 μ L ssG-DNA with regression coefficient (R^2) 0.9782 (Fig. 1[C]).

The specificity of the impedimetric nanohybrid DNA sensor with *S. pyogenes* and other possible bacteria found in human throat swab (*E. coli*, *E. aerogenes*, *B. sphaericus* and *S. aureus*) is shown in Fig. 2. The R_{ct} of the sensor

Scheme 1 Schematic fabrication of gold-PAMAM nanohybrid impedimetric DNA sensor for detection of *S. pyogenes*

Fig. 1 [A] EI spectra of (a) Au/MPA/PAMAM-ssDNA and (b–g) hybridization with 0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 ng/6 μ L of *S. pyogenes* ssG-DNA using redox indicator 5 mM $K_3[Fe(CN)_6]$ and 5 mM $K_4[Fe(CN)_6]$ (1:1) in 50 mM PBS, pH 7. The inset [B] shows hyperbolic curve between relative R_{ct} (with respect to probe as zero) and increasing concentrations of hybridizing ssG-DNA *S. pyogenes*. The inset [C] shows 0–0.1 ng/6 μ L ssG-DNA region of the linear standard graph for calculation of limit of detection and concentration of unknown DNA sample for confirmation of the disease

Fig. 2 Specificity of the Au nanohybrid DNA sensor with *S. pyogenes* and other possible pathogens found in the throat swab of suspected RHD patients. The *inset* shows the relative R_{ct} value (with respect to the immobilized probe as zero) after hybridization with ssG-DNA with other possible pathogens (1.0 ng/6 μ L) and *S. pyogenes* (0.1 and 1.0 ng/6 μ L)

after hybridization with 1.0 ng/6 μ L of ssG-DNA with other bacteria present in throat swab were almost same as the probe except with *S. pyogenes* which shows higher R_{ct} even at lower concentration (0.1 ng/6 μ L) after

hybridization with ssG-DNA (Fig. 2 inset). The significant increase in R_{ct} value was obtained only in the case of *S. pyogenes*, which confirms the specificity of the nanohybrid sensor only to *S. pyogenes*.

The Au nanohybrid electrode was characterized at different stage of modifications using Fourier Transform Infra-Red Spectroscopy (FTIR) in % transmittance mode of peaks at varying wavenumbers (Supplementary material, Fig. S1).

Acknowledgments Ms. Swati Singh thanks Council of Scientific and Industrial Research, New Delhi for providing financial assistance (CSIR-SRF) to carry out the research work. Authors also thank to Prof. N. N. Mathur (ENT), Safdarjung Hospital for providing patient samples.

Compliances with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Guilherme L, Köhler KF, Postol E, Kalil J (2011) Genes, autoimmunity and pathogenesis of rheumatic heart disease. *Ann Pediatr Cardiol* 4:13–21. doi:10.4103/0974-2069.79617
- Dajani AS, Ayoub EM, Bierman FZ, Bisno AL, Deny FW et al (1993) Guidelines for the diagnosis of rheumatic fever: Jones criteria. *Circulation* 87:302–307. doi:10.1002/14651858.CD002227
- Carapetis JR, McDonald M, Wilson NJ (2005) Acute rheumatic fever. *Lancet* 366:155–168. doi:10.1016/S0140-6736(05)66874-2
- Guilherme L, Faé K, Oshiro SE, Kalil J (2005) Molecular pathogenesis of rheumatic fever and rheumatic heart disease. *Expert Rev Mol Med* 7:1–15. doi:10.1017/S146239940501015X
- Pedro MA, Rosa RP, Luiza G (2012) Understanding rheumatic fever. *Rheumatol Int* 32:1113–1120. doi:10.1007/s00296-011-2152-z
- Kalia VC, Kumar R, Kumar P, Koul S (2016) A genome-wide profiling strategy as an aid for searching unique identification biomarkers for *Streptococcus*. *Indian J Microbiol* 56:46–58. doi:10.1007/s12088-015-0561-5
- Kumar R, Koul S, Kumar P, Kalia VC (2016) Searching biomarkers in the sequenced genomes of *Staphylococcus* for their rapid identification. *Indian J Microbiol* 56:64–71. doi:10.1007/s12088-016-0565-9
- Kalia VC, Kumar P (2015) Genome wide search for biomarkers to diagnose *Yersinia* infections. *Indian J Microbiol* 55:366–374. doi:10.1007/s12088-015-0552-6
- Kalia VC, Kumar P, Kumar R, Mishra A, Koul S (2015) Genome wide analysis for rapid identification of *Vibrio* species. *Indian J Microbiol* 55:375–383. doi:10.1007/s12088-015-0553-5
- Kekre A, Bhushan A, Kumar P, Kalia VC (2015) Genome wide analysis for searching novel markers to rapidly identify *Clostridium* strains. *Indian J Microbiol* 55:250–257. doi:10.1007/s12088-015-0535-7
- Koul S, Kalia VC (2016) Comparative genomics reveals biomarkers to identify *Lactobacillus* species. *Indian J Microbiol* 56:253–263. doi:10.1007/s12088-016-0605-5
- Koul S, Kumar P, Kalia VC (2015) A unique genome wide approach to search novel markers for rapid identification of bacterial pathogens. *J Mol Genet Med* 9:194. doi:10.4172/1747-0862.1000194
- Uhl JR, Patel RB (2016) Fifteen-minute detection of *Streptococcus pyogenes* in throat swabs by use of a commercially available point-of-care PCR assay. *J Clin Microbiol* 54(3):815–817. doi:10.1128/JCM.03387-15
- Singh S, Kaushal A, Khare S, Kumar A (2014) Gold–mercapto-propionic acid–polyethylenimine composite based DNA sensor for early detection of rheumatic heart disease. *Analyst* 139:3600–3606. doi:10.1039/C4AN00324A
- Singh S, Kaushal A, Khare S, Kumar A (2014) *mga* genosensor for early detection of human rheumatic heart disease. *Appl Biochem Biotechnol* 173:228–238. doi:10.1007/s12010-014-0836-z
- Dash SK, Sharma M, Khare S, Kumar A (2012) Quick diagnosis of human brain meningitis using *Omp85* gene amplicon as a genetic marker. *Indian J Microbiol* 53(2):238–240. doi:10.1007/s12088-013-0371-6
- Hui P, Lijuan Z, Christian S, Jadranka TS (2009) Conducting polymers for electrochemical DNA sensing. *Biomaterials* 30:2132–2148. doi:10.1016/j.biomaterials.2008.12.065
- Kumar A, Dash SK, Sharma DP, Suman (2012) DNA based biosensors for detection of pathogens. In: Singh HP, Chowdappa P, Chakroborty BN, Podie AR (eds) *Molecular approaches for plant fungal disease management*. Westville Publishers, Delhi, pp 31–35
- Sheehan J, Cruickshank P, Boshart G (1961) A convenient synthesis of water-soluble carbodiimide. *J Org Chem* 26:2525–2528. doi:10.1021/jo01351a600
- Yang T, Guo X, Ma Y, Li Q, Zhong L, Jiao K (2013) Electrochemical impedimetric DNA sensing based on multi-walled carbon nanotubes-SnO₂-chitosan nanocomposite. *Colloids Surf B Biointerfaces* 107:257–261. doi:10.1016/j.colsurfb.2013.01.046
- Park JY, Park SM (2009) DNA hybridization sensors based on electrochemical impedance spectroscopy as a detection tool. *Sensors* 9:9513–9532. doi:10.3390/s91209513