Appl Biochem Biotechnol
DOI 10.1007/s12010-014-1319-y

Surface Plasmon Resonance Based Label-Free Detection of *Salmonella* using DNA Self Assembly

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Received: 26 August 2014 / Accepted: 15 October 2014

Abstract Typhoid is re-emerging as a biggest health threat to third world countries. One of the major challenge is false negative diagnosis using existing immunodiagnostic methods due to overlapping symptoms of other infections (like brucellosis, malaria, hepatitis) that mimic this enteric fever (typhoid). Surface plasmon resonance (SPR) based DNA hybridisation biosensor has been fabricated by generating self-assembled monolayer of 5′-thiolated single-stranded DNA (ssDNA) probe onto gold surface. Highly specific DNA probe has been selected from conserved Vi capsular antigen gene of *Salmonella enterica* serovars *typhi*. This DNA biosensor has been investigated for label-free real-time monitoring of *Salmonella*. Interestingly, 0.019 ng mL^{-1} (2 fM) is the lowest detected concentration in PBS with association (k_a) and dissociation (k_d) rate constants to be k_a (M^{-1} s^{-1})=$6.68\times10^4\pm2.3$ and k_d (s^{-1})=$5.6\times10^{-3}\pm$ 0.01, respectively. This biosensor was successfully demonstrated to distinguish complementary, non-complementary and one-base mismatch sequences and reusability up to 40 hybridisation cycles at room temperature. Successful results were obtained for hybridisation studies of genomic DNA isolated from spiked urine sample. Performance characteristics of this biosensing device suggested further scope to fine-tune such DNA-based assays to be implied for clinical, food and environmental applications.

Keywords *Salmonella* · Surface plasmon resonance · DNA self assembly · Biosensor · DNA probe

Introduction

Globalisation has increased the frequency of international travel/trade among countries and has also facilitated transfer of foodborne pathogens to a greater extent. *Salmonella* sp. is one of the most frequently occurring food/water borne pathogen causing zoonotic infections and has become a significant public health concern worldwide. *Salmonella* (*S.*) *typhi* is an exclusive human pathogen, causing typhoid which is an acute, life-threatening

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Published online: 13 November 2014

febrile illness [1]. Typhoid generally occurs due to consumption of contaminated food or water and exposure to unhygienic sanitation systems. Salmonella infection can lead to generation of symptoms after gestation period of more than a week. Besides this, asymptomatic people infected with this bacterium (called carriers) had been major reason of bacterial transmission to healthy individuals e.g. Typhoid Mary [2]. Human *Salmonella* infections are generally age-specific and affect mostly children, elderly people and immunologically compromised patients [3]. Estimates of the yearly incidences of foodborne illness vary widely from several million cases to 76 million cases in the USA alone, and amongst these, about 91 % of the total outbreaks are of bacterial origin [4]. Monitoring and diagnosis of *Salmonella* is of a great concern not only to the food industry, public and regulatory agencies for quality control purpose but also to clinical diagnostics industry because of overlapping diverse symptoms throughout the world [5]. However, the challenges for bacterial diagnosis are even higher in developing countries due to the absence of appropriate diagnostic methods, hygiene, medical facilities, etc. leading to higher mortality rate (5 to 30 %) in typhoid-infected individuals [6].

World Health Organisation (WHO) has identified more than 2,500 serotypes of *Salmonella* till date, and various methods have been developed for the detection of *Salmonella* spp. These include conventional culture methods that are highly specific for microbial detection where labour-intensive processes of culturing, sub-culturing and phenotypic analysis take 2–3 days for results and up to 7–10 days for confirmation [7, 8], whereas faster contemporary methods utilising antibody-antigen based immunoassays and polymerase chain reaction methods are quite expensive, require several hours and trained personnel, and are less reliable [9–11]. Serological methods like Widal and Secard are still the preferred methods for typhoid detection till date [12], despite their major limitation for its implication at advanced stage of infection (i.e. measuring antibody titre with at least fourfold rise in sera taken at interval of 7 to 10 days) [13, 14]. Moreover, questionable reliability of widal test since last 100 years is due to interference of infections like brucellosis, malaria, and hepatitis that mimics this enteric fever [15]. Therefore, improved alternatives are desired to be developed for reliable sensing of *S. typhi* in clinical and environmental (including food and water) samples.

Advances in biosensors have enabled the development of highly sensitive devices for detection of various analytes of interest. These biosensors comprise of biological recognition component (such as receptors, nucleic acids or antibodies) in intimate contact with an appropriate transducer (electrochemical/optical/mass/thermal) through immobilisation matrix. A biorecognition event occurring at transducer surface can be detected via monitoring various parameters/phenomena. Surface plasmon resonance (SPR) is an optical phenomenon that has been increasingly utilised to develop portable label-free online monitoring system for biorecognition event or binding of analyte onto transducer. SPR relies on the changes in the refractive index (RI) induced by molecular binding onto the interface that can both do qualitative and quantitative estimation of mobile reactant/analyte to its counter partner immobilised on the sensor surface. This can facilitate estimation of binding affinities/kinetic constants of reversible interactions between biological macromolecules in real time [16–20].

SPR has been used in interaction studies and the screening of a variety of biorecognition molecules including proteins, carbohydrates, cells, nucleic acids and receptors leading to applications in areas such as clinical diagnostics, military defence and pharmaceutics [17]. There are various reports that have successfully demonstrated application of SPR for the sensing various analytes including cells or microbial pathogens. Barlen et al. have reported SPR-based sandwich immunoassay for simultaneous and specific detection of *Salmonella*

serovars typhimurium and enteritidis detecting up to 2.50×10^5 cells mL^{-1} and 2.50×10^8 cells mL^{-1}, respectively [21]. SPR based DNA hybridisation detection for synthetic and PCR-amplified DNA samples has been performed using gold-deposited porous anodic alumina (PAA) layer chip up to 10 pM target concentration [22]. A fibre optic SPR sensor has been reported as a reusable, cost-effective and label-free biosensor for measuring DNA hybridisation and DNA–protein interactions using self-assembled monolayer of polyethylene glycol giving 2 nM detection limit [23]. *Escherichia coli* DNA ligase activity assay has been reported using SPR biosensor through hairpin DNA probe detecting up to 0.6 nM concentration [24]. An aptasensor has been demonstrated for amplified SPR based DNA biosensors and Hg^{2+} sensors using Hemin/G-Quadruplexes and Au nanoparticles for DNA detection up to 1 fM [25].

Keeping in view the compromising performance of immunological assays for typhoid detection, DNA based detection is one of the important option available for pathogen detection. DNA is a specific macromolecule present in every cell and known to have interesting physico-chemical properties that can be used to fabricate biosensors. With the change in temperature and pH, two strands of the DNA double helix can be separated and annealed when the conditions are slowly brought to normal. This DNA hybridization can be used for affinity detection of specific complementary DNA [26, 27]. These interesting properties encourage its use for development of nucleic acid based affinity biosensors or genosensors. These genosensors are emerging as an affordable, rapid and easy method for detection that consists ssDNA probe immobilised on the transducer surface which is used to detect affinity interaction with the complementary DNA strands present in the target sample [22, 28]. A key issue with the fabrication of DNA hybridisation biosensor is the immobilisation amount of DNA probe and its accessibility to the target. In this context, self-assembled monolayers (SAM) are increasingly used as immobilisation method for the fabrication of biosensors [30].

SAM is a two-dimensional organised layer of amphiphilic molecules formed at substrate surface through chemical groups that possess strong affinities for the substrate. The entire assembly is strengthened interactions between molecules - substrates and between molecule -molecule via hydrogen bonding, ion pairing and covalent bonds. Particularly well-studied SAM are those formed on transition metal surfaces (e.g. Au, Ag, Cu) and surfactants with electron-rich head groups (e.g. S, O, N) and alkyl tails. The affinities between the surfaces and head groups are strong enough to form either polar covalent or ionic bonds, with sufficiently favourable lateral interactions between adjacent molecules to hold assembly. Therefore, a thiol modified ssDNA probe can be directly immobilised onto bare gold disc used in SPR. Cyclodextrin SAM has been used in SPR based sensor to perform enantioselective analysis of thyroxine [31]. Self-assembled monolayer and nano gold particles mixtures have been reported in SPR assay for small molecules (progesteron) with a detection limit of 4.9 ng L^{-1} [32]. Gobi et al. have demonstrated self-assembled PEG monolayer based SPR immunosensor for label-free detection of insulin. The sensitivity of the prepared immunosensor obtained was 1 ng mL^{-1} (ppb) [33]. Further, they have reported self-assembled monolayer based SPR immunosensor for detection of benzaldehyde using a single-step multi-sandwich immunoassay up to 50 ppt (pg mL^{-1}) [34]. Frasconi et al. have shown use of self-assembled β-cyclodextrin (β-CD)-derivative monolayer (β-CD-SAM) for development of electrochemical surface plasmon resonance (ESPR) study of their inclusion complexes with glucocorticoids with up to 20 μM LOD [35].

In this study, the self-assembled monolayer of 5′-thiol modified ssDNA probe (from conserved Vi capsular antigen gene of *Salmonella enterica* serovars *typhi*) has been prepared for the development of surface plasmon resonance based biosensor for detection of typhoid and salmonella carriers (Scheme 1 and 2).

Experimental

Reagents and Chemicals

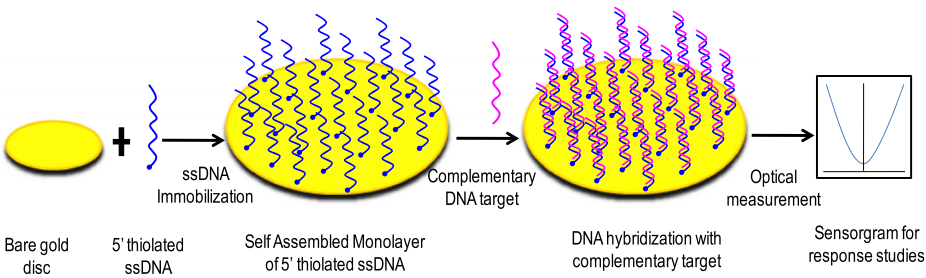
All experiments have been performed using molecular biology grade reagents. Desired buffers and reagents were prepared in de-ionised water (Millipore Express 0.22 μM). All glasswares, pipette, tips and solutions were autoclaved prior to being used. All experiments were performed wearing gloves. 5′-thiol modified DNA probe specific to *S. typhi*, complementary target sequence, non-complementary sequence, one-base mismatch sequence, tris buffer, ethylene diamine tetra acetate (EDTA), H_2O_2, ethanolamine, hydrochloric acid and 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Sigma-Aldrich, USA. H_2SO_4 purchased from Fisher Scientific. Gold-coated BaK7 glass discs have been procured from Metrohm India Pvt Ltd.

Identification of Specific Conserved Sequences of *S. typhi*

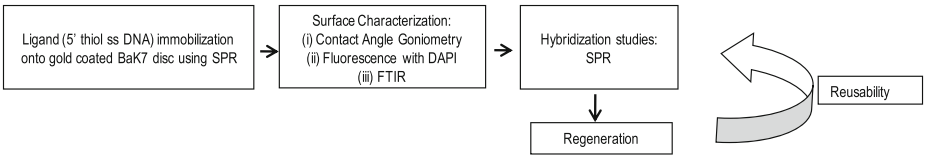
ssDNA probe sequence specific to *S. typhi* has been identified from conserved region Vi capsular antigen gene, part of *S. enterica* serovars *typhi* using online database search provided by NCBI blast search. This sequence has been tested through BLAST (blast.ncbi.nlm.nih.gov) search, and it has been found that probe sequence does not match to any other related species of family Enteriobacteriaceae as well as with other micro-organisms. DNA sequences used in present study are listed in Table 1

Preparation of Biosensor Disc

Prior to immobilisation of thiolated DNA probe, gold discs were treated with piranha solution (3:1 mixture of ammonium hydroxide with hydrogen peroxide) followed by rinsing with de-ionised water and subsequent ultrasonication in absolute ethanol (for about 2 min) along with consecutive rinsing with de-ionised water. DNA probe was suspended in TE buffer [Tris (10 mM) and EDTA (1 mM)] pH 8.0, and this stock solution was kept at −20 °C when not in use. The 5′-thiol modified



Scheme 1 Fabrication of SPR based optical DNA biosensor



Scheme 2 Methodology for fabrication, optimisation, characterisation and response studies of the DNA biosensor for detection of *Salmonella typhi*

DNA probe resuspended in autoclaved de-ionised water and immobilised onto the pre-cleaned gold surface at room temperature as shown in Scheme 3, and the immobilisation of the molecules was monitored using SPR spectroscopy for about 800 s.

Results and Discussion

Immobilisation of DNA

Figure 1 shows SPR real-time sensorgram obtained for the immobilisation of 5′-thiol modified ssDNA probe specific to *S. typhi*. It can be seen that the first phase of 120 s shows baseline with de-ionised water, after which solution of thiolated DNA is introduced for immobilisation up to 800 s onto gold coated onto BaK7 glass disc at room temperature, followed by a washing step.

The change in the SPR angle (or gradual increase in the angle with time) corresponds to binding of ssDNA at the Au surface. This step was followed by the introduction of de-ionised water to remove the unbound ssDNA. It has been found that there is a change of 412 millidegrees (m^o)at the end of immobilisation phase (i.e. at 900 s as shown in Fig. 1) corresponding to 3.43 ng mm^{-2} DNA bound to the surface.

Surface Characterisation of DNA-Au Biosensor

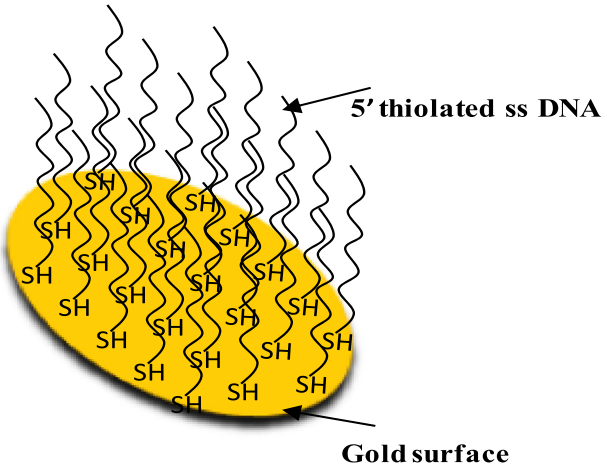
5′-thiolated ssDNA probe immobilised onto the BaK7 gold disc has been characterised using contact angle measurement (CAG), fluorescence microscopy (FM) and Fourier transform infrared spectroscopy (FTIR) as discussed below:

Contact Angle Measurements

Contact angle studies of bare gold disc and ssDNA/Au are shown in Fig. 2. Contact angle of bare gold has been found to be 76° (Fig. 2a), and for ssDNA/Au, it is 63° (Fig. 2b). The decrease in contact angle values after immobilisation of ssDNA probe can be attributed to

Table 1 Single-stranded DNA probe sequences for detection of S. *typhi*

Nucleic acid probe	Sequence
DNA probe	5′[Thiol-C6] CGTGCGCGACGCCCGCCGCC-3′
Complementary target	5′-GGCGGCGGGCGTCGCGCACG-3′
One-base mismatch	5′-GGCGGCGGGCG**A**CGCGCACG-3′
Non-complementary	5′-GTAACCTGCCTGTAAGACTG-3′



Scheme 3 SAM of 5′-thiol end modified ssDNA onto gold-coated BaK7 glass discs

increase in hydrophilicity onto surface of Au which is due to introduction of NH_2 and OH groups present in DNA, therefore indicating the successful immobilisation/binding of ssDNA probe onto Au surface which is in correlation with the SPR studies (Fig. 1).

Fluorescent Microscopic Studies

Figure 3 shows the fluorescent images of bare Au surface, ssDNA/Au and dsDNA/Au surface after staining with DAPI using violet filter. DAPI or 4′,6-diamidino-2-phenylindole is a fluorescent stain that binds strongly to A-T rich regions in the DNA. When bound to double-stranded DNA, DAPI has an absorption maximum at a wavelength of 358 nm (ultraviolet), and its emission maximum is at 461 nm (blue). Therefore, during this processing

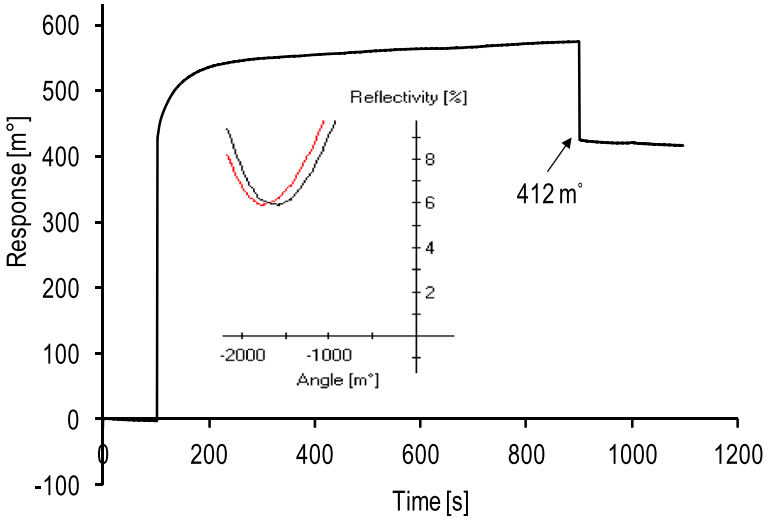


Fig. 1 SPR sensorgram showing covalent immobilisation of 5′-thiolated ssDNA

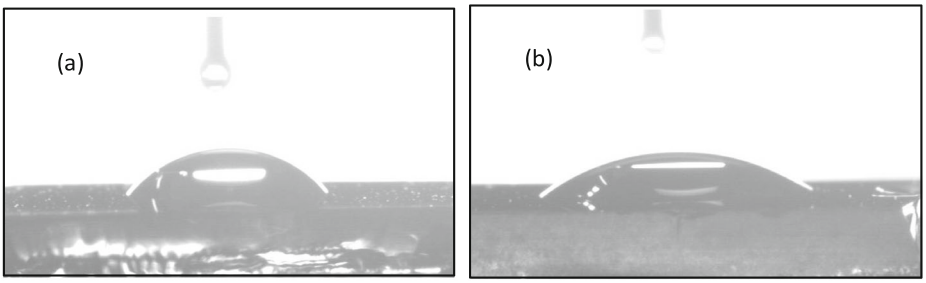


Fig. 2 Contact angle measurement of **a** bare gold disc and **b** ssDNA/Au surface

for fluorescence microscopy, DAPI is excited with ultraviolet light and is detected through a blue/cyan filter. It can be seen that no fluorescence has been observed for bare Au surface (Fig. 3a) and ssDNA/Au (Fig. 3b), whereas ds DNA/Au surface (Fig. 3c) is showing fluorescence signal in the U-MWU2 (violet excitation 330–380 nm; emission 420 nm) detected by the camera DP71 (resolution 12.5 megapixel).

Although the DNA sequence comprises only 2 AT pairs, the fluorescence signal is enough to indicate that the fluorescence thus obtained through violet filter can be attributed by the presence of double-stranded DNA.

FTIR Studies of ssDNA/Au Biosensing Surface

The FTIR spectrum of the ssDNA/Au and bare gold surface was performed on Niconet 6700 FTIR machine as shown in Fig. 4, from 500 cm^{-1} to 1,700 cm^{-1} at room temperature. No significant peak was observed for bare gold surface (curve i). Peaks at 688 cm^{-1} (curve ii) can be attributed to P-C stretching present in sugar-phosphate backbone of DNA i.e. between phosphate group and carbon of deoxyribose sugar confirming the immobilisation of ssDNA onto bare gold disc [36, 37].

Hybridisation Studies Using Surface Plasmon Resonance

Hybridisation studies have been carried out using the SPR technique. In each SPR experiment, the SPR signal with the DNA/Au biosensing surface is first recorded with de-ionised water for 120 s to obtain the baseline value, after which the solution of complementary sequence is added and is allowed to interact with the biosensing surface for the next 800 s (association

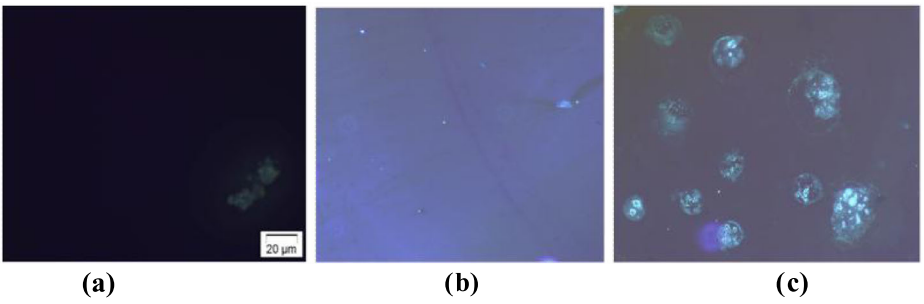


Fig. 3 Fluorescent images of **a** bare gold surface, **b** ssDNA/Au and **c** ds DNA/Au through violet filter. Background flourescence is due to increased exposure time

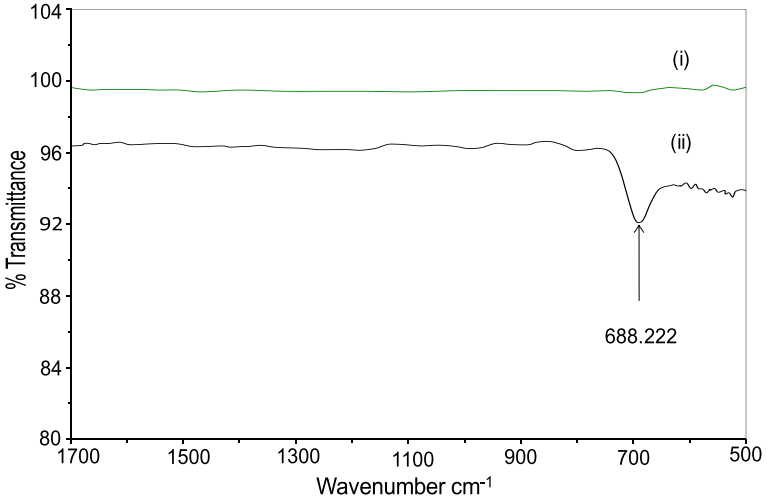


Fig. 4 FTIR spectra. Bare gold disc (*green*; *i*) and ssDNA/Au biosensing surface (*black*; *ii*) (*colour figure online*)

phase or nucleic acid hybridisation). On completion of the association phase, the unused solution is discarded, and the biosensing surface is washed with de-ionised water (dissociation phase). After this, the SPR signal with de-ionised water is recorded, and the change in the SPR angle is measured. This change in the SPR angle before and after the association phase corresponds to the amount of binding while keeping all other parameters constant.

Hybridisation detection was carried out to detect the binding of complementary target sequences with the single-stranded DNA probe immobilised onto gold disc for *S. typhi*. It has been found that binding/hybridisation occurs with complementary target sequences. However, slight change was observed in the case of one-base mismatch sequences, and this is due to the fact that mismatch formed on the distal end of duplex is more flexible and more accessible by positive ions, and the electrostatic repulsion among DNA strands is reduced by the presence of positive ions [38], while no binding/hybridisation was observed for non-complementary sequences as shown in Fig. 5. Results show that prepared biosensor is able to distinguish between complementary, non-complementary and one-base mismatch sequence.

S. typhi ss DNA immobilised gold discs have also been used to study the influence of concentration of complementary targets. It has been found that upon decreasing the concentration of the complementary target significant change in reflectance angle (m^o) observed from 40 to 2 fM. Figure 6 shows improved detection limit (~500 times) compared to literature (Table 2) [39-44]. Kinetic calculations have been carried out for complementary target having association constant and dissociation constant as k_a (m^{-1} s^{-1})=$6.68\times10^4\pm2.3$ and k_d (s^{-1})= $5.6\times10^{-3}\pm0.01$, respectively. Besides this, it may be noted that the same DNA probe immobilised surface was regenerated using 0.05 M NaOH and the biosensing surface was reutilised for detection of complementary target for about 40 times.

As prepared DNA biosensor has been studied to check the influence of serum on DNA hybridisation. It has been observed that a significant rise in angle is attributed to high mass transport due to proteins present in serum (Fig. 7). This mass transport influenced the actual concentration of complementary target which may result in wrong interpretation of results, thereby questioning its implementation for further

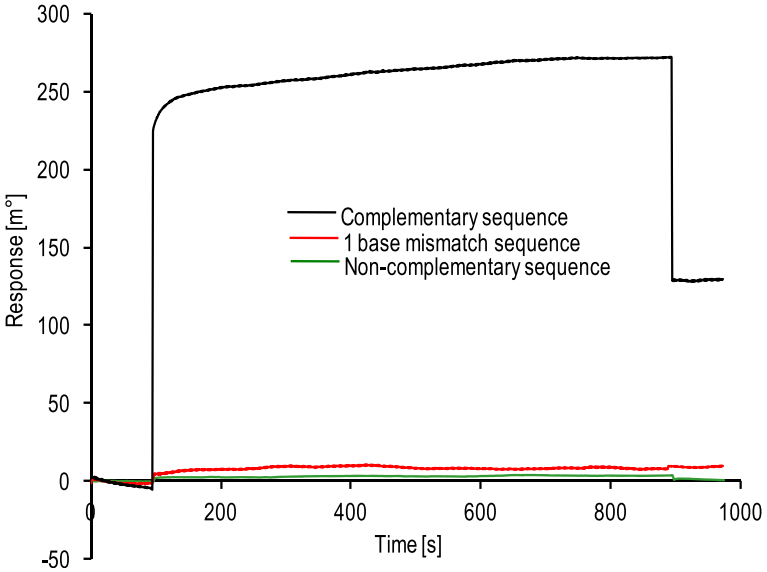


Fig. 5 Response curves of *S. typhi* probe DNA immobilised gold disc using SPR after hybridisation with (i) complementary target (129 m°, *black*), (ii) one-base mismatch target (6 m°, *red*) and (iii) non-complementary target (*green*) (colour figure online)

analysis. To utilise the available SPR spectrometer's niche and to avoid serum related complications, urine has been investigated hereafter instead of serum/blood.

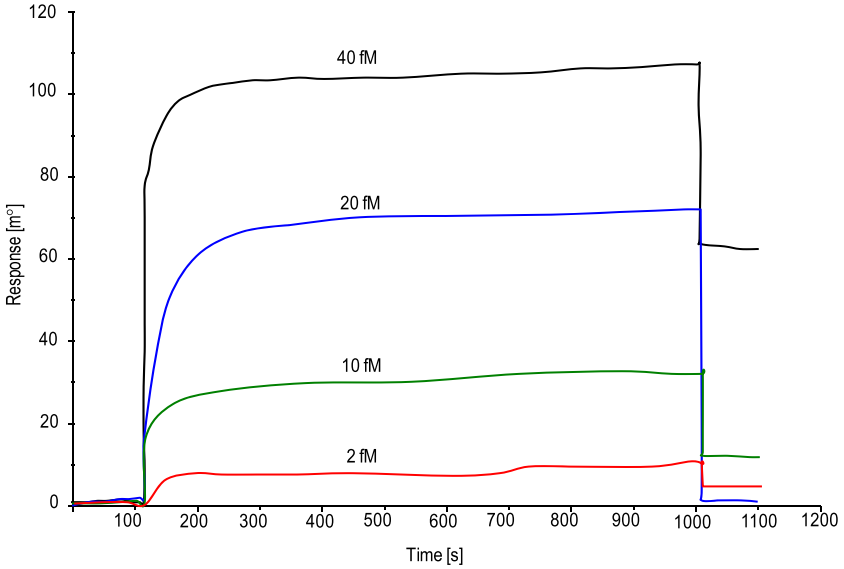


Fig. 6 Response curves of *S. typhi* single-stranded probe immobilised gold coated glass disc for various concentrations of complementary targets [40 fM (*black*), 20 f. (*blue*), 10 fM (*green*) and 2 fM (*red*)] (colour figure online)

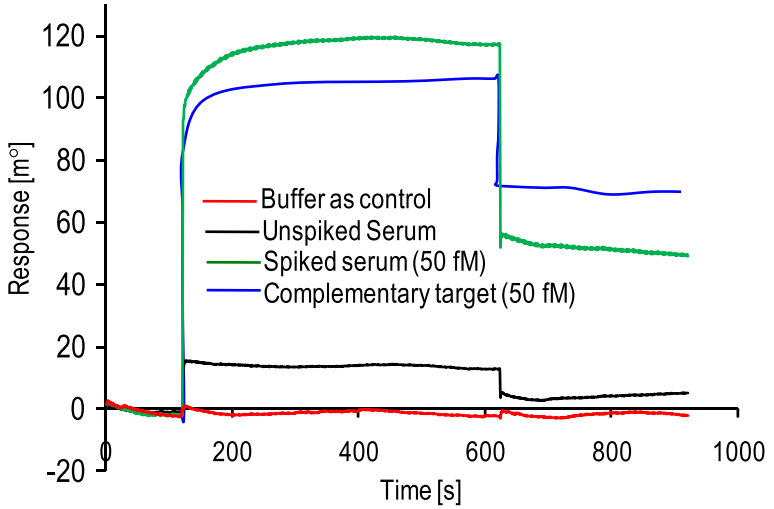


Fig. 7 Sensorgram showing influence of serum on DNA hybridisation

Response Studies of DNA/Au Biosensing Surface in Culture Spiked Urine Sample

The performance of DNA/Au biosensing surface has been investigated by spiking bacterial culture (*S. typhi*) in the urine of a normal person. Five millilitre volume of urine sample from a healthy individual has been spiked with 100 μL of bacterial culture (*S. typhi* and *S. paratyphi A*) i.e. ~6×10^9 CFU mL^{-1} at 1/10 OD of 0.8, calculated using serial dilution method. The urine sample was centrifuged at 2,000 rpm for 20 min to obtain bacterial cells that are re-suspended in 200 μL buffers (50 mM Tris and 10 mM EDTA, pH 7.5) and sonicated for 3 min to obtain fragmented DNA strands. Further, 10 % TCA in acetone (chilled) was added, vortexed and incubated at −20 °C for 3 h to remove proteinaceous contamination. The solution was then centrifuged at 15,000 rpm for 30 min, and supernatant was collected for further DNA hybridisation detection. Total DNA extracted is denoted as N.

SPR studies of DNA/Au biosensing surface (for *S. typhi*) have been carried out using various dilutions of extracted genomic DNA i.e. N, N/2, N/4, N/8. In Fig. 8a, SPR sensorgram obtained for genomic DNA dilutions is showing gradual increase in response signal with time. Hybridisation time has been increased for 2,000 s (i.e. around 33 min) to facilitate maximum binding of complementary sequences present in the sample. Specificity of the prepared surface has been checked by extracting genomic DNA of other organisms (*E. coli* and *S. paratyphi A*) using the same procedure and then hybridising the obtained genomic DNA with the sensing surface. In Fig. 8b, SPR sensorgram shows that nominal rise has been obtained for non-specific genomic DNA which may be attributed to non-specific hybridisation of some very small fragments. However, compared to *S. typhi*, this rise in hybridisation is insignificant, and DNA/Au biosensing surface is showing good ability to discriminate specific and non-specific genomic samples.

Regeneration of the DNA/Au Biosensing Surface

After the completion of the dissociation phase, the surface is fully regenerated by washing with HCl solution (2.0 mM) in the regeneration phase (nucleic acid

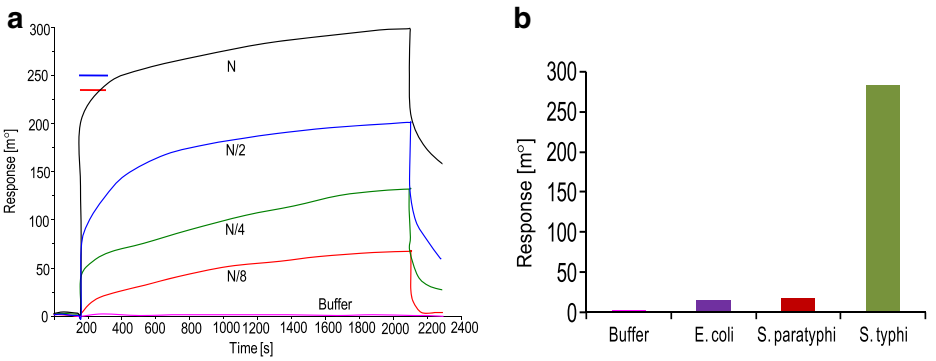


Fig. 8 **a** SPR sensorgram showing hybridisation with various dilutions of isolated genomic DNA of *S. typhi*. **b** Bar graph obtained for hybridisation studies with isolated genomic DNA of non-specific targets

denaturation) for 300 s. To optimise the regeneration procedure, different concentrations of HCl and NaOH were tested. Among them HCl showed higher regeneration capacity at the optimised concentration of 2.0 mM. It was found that single-stranded probe can be regenerated by 5 min treatment with 0.2 mM HCl and is then ready for a new hybridisation cycle.

Stability, Repeatability and Reusability Studies

The ssDNA/Au biosensing surfaces were kept at 4 °C under desiccated condition, and the hybridisation studies were carried out after every 7 days to check the response characteristics of the biosensing surface. To attest the repeatability results of the ssDNA/Au biosensing surface, the same concentration of the complementary target was hybridised and the response signal was measured in terms of change in m° for at least five times. Reusability studies were carried out via regenerating the biosensing surface by use of 0.2 mM HCl as mentioned earlier. Change in m° was plotted against the number of times, and the same ssDNA/Au surface was reutilised for hybridisation.

Conclusion

SPR based DNA biosensor has been fabricated using self-assembled monolayer of single-stranded 5′-thiol modified DNA probe (specific to *Salmonella*) onto BaK7 gold disc. This fabricated ssDNA/Au has been used for online, label-free, specific detection of complementary target up to 0.019 ng mL^{-1} (2 fM) showing improvement of ~500 times. Besides this, the electrode has been reused for about 40 times. Spiked urine samples have been successfully demonstrated for identification of *S. typhi* in urine through DNA based detection without using PCR amplification and stringent washings. These results open up new opportunities for online diagnosis of Salmonella carriers. Efforts are being made to improve the characteristics of DNA/Au biosensing surface to directly detect the complementary sequence present in clinical samples. These investigations spot light important results having vast applications in the field of online detection of microbes in various mediums of clinical, food and environmental interest.

Table 2 Detection limit of fabricated DNA biosensor in comparison with reported literature

Serial no.	Sample	Matrix	Transducer	Detection limit	Reusability	Response time	Reference
1.	*Salmonella paratyphi*	SAM protein G	SPR	10^2 CFU mL^{-1}	–	–	[39]
2.	*E. coli*	Avidin-modified polyaniline	Electrochemical	9.0 ng mL^{-1}	5 - 7 times	60 s - 14 min	[40]
3.	*E. coli*	Streptavidin-biotin	SPR	1.9×10^4 CFU mL^{-1}	25 times	–	[41]
4.	*M. tuberculosis*	5′-thiol-end-labelled DNA	SPR	10 ng mL^{-1}	–	–	[42]
5.	*Staphylococcal enterotoxin* A	Mixed SAM (16MHA:6MHA)	SPR	100 - 1,000 ng mL^{-1}	–	–	[43]
6.	Synthetic and PCR-amplified DNA samples	Gold-deposited porous anodic alumina (PAA) layer chip	SPR	10 pM	–		[22]
7.	300-bp PCR products	5′-thiol labelled DNA	SPR	2.6 nM	–	5 min	[44]
8.	*Salmonella typhi*	5′-thiol labelled DNA	SPR	2 fM	40 times	60–150 s	Present study

Acknowledgments We are grateful to Prof. Sopory, Vice Chancellor, Prof. Subhasis Ghosh, Director, AIRF, JNU, India, for their constant encouragement and infrastructural support. Thanks are due to Ms. Meenakshi and AIRF team members for their kind support and cooperation. DBT-sponsored projects IYBA-2008 (BT/B1/12/045/2008) and RGYI-2009(BT/PR13127/GBD/27/195/2009), UPE1- capacity buildup fund, DST PURSE are dully acknowledged.

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