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Electrochemical biosensor for serogroup specific diagnosis of leptospirosis

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ABSTRACT

A problem with the current leptospirosis diagnostic methods is the low sensitivity and specificity during the acute phase of illness. Rapid point-of-care (POC) assays with minimal sample utilization and low cost are desired in clinical practice. Here, we report for the first time lipopolysaccharide (LPS) based electrochemical biosensor that offers a rapid, highly sensitive, serogroup specific diagnosis of leptospirosis during the acute stage of infection and also to distinguish from other flu like infections. The proposed sensor is fabricated by the immobilization of LPS onto dodecanethiol (DT) modified gold electrode. Monolayer of DT is attached through covalent bond (Au-S) interaction onto the gold electrode. Thus, leptospiral antibodies from the human serum samples bind to the LPS present on self-assembled monolayer (SAM) of DT and showed a higher R_{CT} value compared to SAM. The detection limit of the developed LPS sensor is estimated to be 100 nM. This biosensor is the first electrochemical sensing platform used for detection of LPS from *Leptospira* spp. This method is completely a solution-based diagnostic method and therefore it is rapid, simple, and sensitive; thus establishing a key technology towards a useful POC diagnostic strategy in serogroup level and hence an alternative to MAT.

1. Introduction

Leptospira is a member of order Spirochaetales and belongs to the family Leptospiraceae, which can be either pathogenic or saprophytic [1]. Pathogenic leptospires are present in the renal tubules of infected or carrier animals [2]. Rodents are the major reservoir of leptospirosis, which is an important emerging global public health problem distributed worldwide. The diagnostic tests incurred costs, between 15.3 and 17.2 USD per patient [3]. Usually the infection rate is higher in the tropical regions than in the temperate region [4]. The clinical manifestations vary from mild flu like illness to multi-organs failure and in serious conditions it may even lead to death of the infected host [5]. However, during the early stage of illness the clinical symptoms of leptospirosis are non-specific and challenging to distinguish from dengue, malaria, influenza, and many other febrile diseases [6]. Hence, laboratory based tests are needed to confirm the diagnosis to initiate proper treatment and that is vital for controlling the morbidity and mortality levels [7]. The incidence of leptospirosis and its associated mortality rates with an increased outbreak identified leptospirosis as a fatal disease worldwide [8].

Abbreviations: **LPS**, **Lipopolysaccharide**; **DT**, **Dodecanethiol**; **MAT**, **Microscopic Agglutination Test**; **ELISA**, **Enzyme-linked immuno-sorbent assay**; **ICG-LFB**, **Immuno-chromatography Lateral Flow Assay**; **MCDA-LFB**, **multiple cross displacement amplification-lateral flow biosensor**; **ET**, **Electron Transfer**; **EIS**, **Electrochemical Impedance Spectroscopy**; **EMJH**, **Ellinghausen-McCullough-Johnson-Harris Medium**; **IEC**, **Institutional Ethics Committee**; **PBS**, **Phosphate Buffer Saline**; **SDS-PAGE**, **Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis**; **kDa**, **Kilo Dalton**; **PPV**, **Positive Predictive Value**; **NPV**, **Negative Predictive Value**; **XPS**, **X-ray Photoelectron Spectroscopy**; **CV**, **Cyclic Voltammetry**; **$K[Fe(CN)_6]^{3-/4-}$**, **Potassium hexacyanoferrate (III & IV)**; **SCE**, **Saturated Calomel Electrode**; **NaF**, **Sodium Fluoride**; BSA, Bovine Serum Albumin; AFM, Atomic **Force** Microscopy; WDCA, Water- Drop Contact Angle; MCAT, Micro Capsule Agglutination Test; Au electrode, Gold Electrode; E/V vs. SCE, Potential with respect to the saturated calomel electrode; Z' (Ω cm2), Real part of impedance; - Z'' (Ω cm2), Imaginary part of impedance; J (A/cm2), Current density; RCT, Charge transfer resistance; RS, Solution Resistance.

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Even though numerous techniques are used for the diagnosis of leptospirosis, performing microscopic agglutination test (MAT) to demonstrate four-fold raise in antibody titre and isolation of leptospires from the infected specimen (blood, urine and/or relevant fluids are employed as target samples) are the direct confirmatory evidences of leptospirosis [9]. Though MAT is deemed as a "gold standard" reference serovar specific test for diagnosis of leptospirosis, it is difficult to perform. Also it involves a large panel of live leptospiral culture as antigen and moreover it needs professional experience and skilled manpower for interpretation of results [10]. As leptospirosis is a critical human health problem in developing or under-developed tropical countries, thus, a precise diagnostic format that can be used for point-of-care analysis is vital. Enzyme-linked immuno-sorbent assay (ELISA), dipstick assay, Immuno-chromatography Lateral Flow Assay (ICG-LFA), multiple cross displacement amplification-lateral flow biosensor (MCDA-LFB) method, macroscopic agglutination test, and microcapsule agglutination are the other diagnostic formats developed for the diagnosis [11,12]. Among these methods, ELISA offers acceptable sensitivity and the option of handling many samples at a time and has been established and evaluated with number of variations starting with the use of crude antigens to conserved peptides etc. [13,14]. A Recent study established the role of circulating stable microRNAs (miRNAs) (miR-21-5p, miR-144-3p, and miRlet-7b-5p) as an early diagnostic marker for leptospirosis. These miRNAs were able to discriminate the acute leptospiral infection from other febrile diseases [15]. However, the above mentioned diagnostic techniques are primarily based on the conserved protein antigens/miRNAs and cannot be employed for serogroup level identification. Till date there is no serogroup specific diagnostic formats to replace the reference, MAT.

Lipopolysaccharide (LPS), a major component present in the outer membrane of *Leptospira* is one of the major essential immuno-dominant antigens. The antigenic nature of LPS initiates the immuno-modulatory activity during pathogenesis and structural variation between serogroup helps in the serogroup level identification of *Leptospira* species [16,17]. Concerning, validation of such serogroup specific antigens in diagnostics can serve as an alternative to MAT. Electrochemical detection is a desirable unconventional technique for application in primary health care diagnosis due to its considerable advantages for a point-of-care testing facility. Electrochemical immunosensor based on gold-labeled monoclonal anti-LipL32, Loa22 gene based DNA biosensor for the diagnosis of leptospirosis was developed and being a genus-specific technique unable to find the infecting serovar [18–20].

In this context, here, for the first time, we attempt to develop an electrochemical biosensor for serogroup level diagnosis of leptospirosis. Electrochemical sensors offer rapid response, higher sensitivity, simplicity and ease to use [21]. Particularly the sensing layer for LPS detection includes a SAM of dodecanethiol (DT), antibodies and functionalized LPS that generates the change in the electronic properties upon binding of LPS to specific antibodies. Binding events are then probed by measuring the electron transfer (ET) reaction associated with the redox-active species in a solution using electrochemical impedance spectroscopy (EIS) [22]. The charge transfer resistance, R_{CT} is determined by fitting the experimental data to a semicircle and determining its intercept on the x-axis based on the modified Randle's equivalent circuit. Later, this parameter is correlated with the binding of target analyte onto the modified electrode surface.

In order to develop a serogroup specific diagnosis, we target to build up leptospiral LPS based electrochemical immunosensor using a gold electrode modified with SAM of DT. The binding events were measured using EIS, and our results reveal that the sensor has higher selectivity and sensitivity to the leptospiral specific homologous sera compared to heterologous and sera from other febrile cases. The analytical performances were evaluated systematically for the developed immuno-sensors for their applicability for a serogroup specific diagnosis of leptospirosis. Proposed LPS based detection strategy allows serogroup specific detection due to the unique structural difference exists between the serogroups. Until now, MAT is considered as gold standard technique for serogroups level identification of leptospirosis, but it is laborious and difficult to maintain live leptospiral cultures all the time for while performing the experiments and inter-laboratory variations may also occur. Hence the proposed LPS based strategy allows one to overcome the drawbacks of MAT and is an ideal for alternative to MAT.

2. Experimental procedures and characterization

2.1. Leptospiral strains and media

A panel of pathogenic leptospiral strains: *Leptospira interrogans* serovar, Australis strain Ballico, *Leptospira interrogans* serovar, Autumnalis strain N2, *Leptospira borgpetersenii* serovar Ballum strain Mus 127, *Leptospira interrogans* serovar Bataviae strain Swart, *Leptospira interrogans* serovar Canicola strain Hond Utrecht IV, *Leptospira kirschneri* serovar Grippotyphosa strain Moskva V, *Leptospira interrogans* serovar Hebdomadis strain Hebdomadis, *Leptospira borgpetersenii* serovar Javanica strain Poi, *Leptospira interrogans* serovar Pomona strain Pomona, *Leptospira interrogans* serovar Sejroe strain Hardjoprajitno, *Leptospira interrogans* serovar Pyrogenes strain Salinem, and the non-pathogenic *Leptospira biflexa* serovar Andamana CH11 were obtained from World Health Organization (WHO) Reference Centre for Leptospirosis, Regional Medical Research Centre, Indian Council of Medical Research (ICMR), Port Blair, India and used for the present study. Strains were maintained by regular sub-culturing in Ellinghausen-McCul lough-Johnson-Harris (EMJH) bovine serum albumin-Tween 80 medium (Difco Laboratories, USA) in a shaking incubator (100 rpm) at 30 °C.

2.2. Patients and case definition

In total, 252 serum samples collected during the early phase of illness (0 to 10 days after the onset of disease) were selected from a bank of 370 laboratory-confirmed cases of leptospirosis (a positive IgM ELISA or MAT titer of $\geq$ 1:160 or isolation of leptospires from the blood). A total of 56 seronegative healthy controls matched with respect to age (±5 years) and sex, and 39 patients who were hospitalized with a clinical suspicion of leptospirosis and subsequently diagnosed as having other illness including dengue (18), malaria (17) and typhoid (04) based on laboratory evidences were also included in the study. Informed written consent was obtained from both the cases and controls before sampling, and the study protocol was approved by the Institutional Ethics Committee (IEC) of Bharathidasan University (BDU/IEC/2020/02; DM/2014/101/49), as well as permitted by the Directorate of Health Services (5796/TV1/07), Tamil Nadu with due approval.

2.3. Extraction of leptospiral LPS, western blotting and ELISA

Leptospires were collected from mid-log cultures by centrifugation at 10,000xg for 30 min and then washed three times with sterile 1X Dulbecco's phosphate-buffered saline (PBS) (Corning, Manassas, VA, USA) before LPS extraction. LPS was extracted following the standard hot phenol-water extraction as described previously [16]. LPS was quantified by the phenol/sulfuric acid method using sucrose as a standard [23]. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) profiling

of extracted was performed on a 12% polyacrylamide gel using a discontinuous buffer system. The extracted LPSs were mixed with 2X SDS-PAGE sample loading buffer (BioRad, Hercules, CA, USA) and boiled for 5 min before loading. Electrophoresis was carried out in a vertical electrophoretic mini-cell unit (Bio-Rad, Hercules, CA, USA) for 2 h at 100 V using Tris-glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) [24]. A modified silver staining procedure was performed to detect the LPS bands on SDS-PAGE [16,25] and documented using a gel documentation system (Bio-Rad, Hercules, CA, USA). The apparent molecular mass of the extracted LPSs lies between 14 and 28 kDa (**Fig. S1A**). The separated LPSs were transferred to nitrocellulose membranes (0.2 μm; Bio-Rad, USA) [24] using V20 semi-dry blotter (Scie-Plas, UK), and probed with serum (1:200) from confirmed cases for leptospirosis, followed by incubation with anti-human IgG antibody conjugated with *horseradish peroxidase* (Sigma-Aldrich, St. Louis, MO, USA) and the bands were visualized by using 4-chloro-α-naphthol (Sigma-Aldrich, St. Louis, MO, USA) (**Fig. S1B**) and it showed the reactivity to respective pathogenic leptospiral LPS. There was no reactivity was observed in non-pathogenic serovar Andamana. Serogroups reactivity was observed for all leptospiral LPSs against their homologous and heterologous sera confirming the serogroups specificity and reactivity. Moreover, sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of homologous sera values obtained by ELISA were represented in Table 1.

2.4. Electrochemical studies

The binding events occur during the sensing of these LPSs for the diagnosis of leptospirosis were monitored using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). All these electrochemical studies were performed with an Autolab PGSTAT 302 instrument (Metrohm, Autolab-Potentiostat/ Galvanostat Instruments (PGSTAT302N), Netherlands). The electrochemical properties of the monolayer towards electron transfer process were studied using $K[Fe(CN)_6]^{3-/4-}$ redox couple as a probe. The electrolyte solutions used for electrochemical measurements were prepared using Milli-Q water (Millipore, Merck Life Science, Darmstadt, Germany) having a resistivity of 18.2 MΩ cm. CV and EIS studies were conducted in a three-electrode system at 25 °C consisting of a platinum rod/ wire, a saturated calomel electrode (SCE) and modified gold electrode as a counter, reference, and working electrodes respectively. Cyclic voltammetry was performed in 1 mM potassium ferro/ferricyanide aqueous solution containing 0.1 M NaF as a supporting electrolyte. Impedance measurements were carried out at the formal redox potential ($E_{1/2}$ = 0.16 V vs. SCE, as determined from cyclic voltammetry) in an aqueous solution containing equal concentrations of both the oxidized and reduced forms of $[Fe(CN)_6]^{3-/4-}$ (1 mM each) with 0.1 M NaF as a supporting electrolyte [26]. The frequency ranging from 100 KHz to 0.01 Hz with an ac amplitude of 5 mV was used for the impedance analysis.

2.5. X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectra were recorded with a MulitiLAB system (Multilab 2000, Twin anode X-ray source, Thermo scientific, UK). Gold-coated plates were used to prepare the samples that were mounted with a spring clip during the measurement. A monolayer of DT was prepared by immersing the cleaned gold strips into neat DT for 1 h. After this, the SAM modified gold surface was thoroughly rinsed with ethanol and Milli-Q water (Millipore, Merck Life Science, Darmstadt, Germany). Then, DT monolayer-modified gold electrode was dipped into an aqueous solution of LPS having a concentration ranging from 20 nM to 100 μM for about 1 h. After this LPS-DT modified gold electrode was rinsed with Milli-Q water. Further, the LPS coated DT monolayer-modified gold electrodes were dipped into an aqueous solution of 100 μM bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) for about 1 h each, after which they were thoroughly rinsed with Milli-Q water. After blocking with BSA, the LPS coated electrodes were dipped into an aqueous solution of either the homologous or the heterologous patient's sera (contain the specific antibodies against the leptospiral LPS) and sera of healthy controls. In a typical experiment, a few survey scans in the binding energy ranging from −10 eV to 1350 eV were collected at a resolution of 1.0 eV. Then, detailed scans of 10 eV – 30 eV over a single feature/ element were collected at a resolution of 0.1 eV. XPS is primarily used to identify the presence of elements along with their corresponding oxidation states and the confirmation of LPS binding onto the modified electrode surfaces.

2.6. Contact angle measurements and Atomic Force Microscopy (AFM) analysis

Water-drop contact angle (WDCA) measurements were also performed at room temperature by employing the sessile drop technique using a commercial VCA Optima instrument. All the contact angle values were determined from the average of at least three independent measurements performed under the same conditions at different locations on the sample surface. Similarly, the surface topography of bare gold electrode, DT monolayer modified Au, leptospiral LPS immobilized DT electrode and the anti-leptospiral antibody functionalized Au electrode was analyzed using Atomic Force Microscopy [AFM] (5500 scanning probe microscope, Agilent Tech., USA) in a non-contact mode. The surface morphological features and thickness profiles were analyzed using the software provided by them.

3. Results

3.1. Construction and characterization of the sensing electrode

Here in, we demonstrated a simple and efficient way to detect *Leptospira* specific antibodies using chemically modified gold elec-

Table 1
Sensitivity, specificity, PPV and NPV of homologous sera.

LPS (Serogroup)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	kappa value
Australis	93.75	99.16	83.30	99.71	0.876
Autumnalis	91.67	100	100	99.43	0.953
Bataviae	100	99.72	92.85	100	0.962
Canicola	96.43	99.68	98.18	99.37	0.968
Grippotyphosa	93.40	99.03	98.80	99.51	0.984
Javanica	92.86	100	100	99.72	0.962
Pomona	93.30	99.71	96.55	99.42	0.945
Pyrogenes	100	99.71	96.00	100	0.978

trodes. The overall construction process is depicted in Scheme 1A-E. Evaluation of surface modification steps and probing of the binding events towards the development of electrochemical biosensor for the detection of leptospirosis is carried out using electrochemical techniques namely cyclic voltammetry and impedance measurements using potassium ferro/ferricyanide as a redox probe. Impedance spectroscopy data were analyzed using an equivalent circuit fitting procedure based on Randle's equivalent circuit model **(Fig. S2A and S2B)** (sometimes modified one) to determine the charge transfer resistance (R_{CT}), a parameter used to quantify the rate of electron transfer across the interface in turn the binding events. **Fig. S2C and S2D** show the CVs and Nyquist plots of bare Au electrode before modification and after different modification steps used for the fabrication of sensor along with the corresponding equivalent circuits used for fitting the measured impedance data and for the determination of parameters like R_{CT}. Here Z' represents the real part and Z" denotes the imaginary part of the impedance at the frequency range between 100 kHz and 0.01 Hz with an alternative current amplitude of 5 mV.

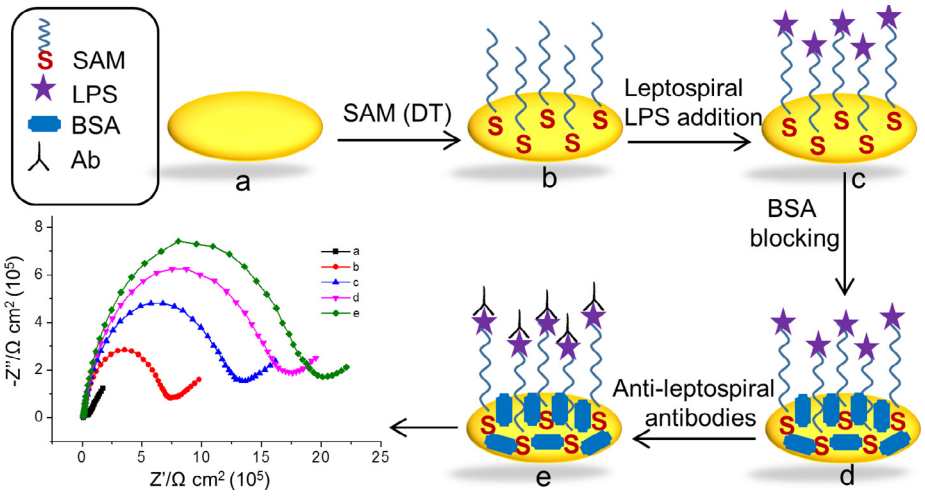
Several factors contribute the electronic or ionic properties of the surface electrode, such as the solution resistance (RS), the double layer capacitance (C) associated with Warburg formed by the ions at the vicinity of the electrode, the charge transfer resistance (RCT) that represents the current flow due to redox reactions at the electrode electrolyte interface upon addition of different element such as LPS, BSA and anti-leptospiral antibodies and a constant phase element Table 2.

To illustrate the surface binding after each modification step, WDCA measurements were performed and the results are shown in **Fig. S2E**. WDCA analysis is used to determine the wettability of the functionalized surfaces. Initially bare Au surface without any modification exhibits a contact angle of 83.20°, while the contact angle of SAM modified surface was measured to be 99.30°, revealing the formation of monolayer of DT leading to hydrophobicity of the resultant modified surface. Further the addition of leptospiral LPS onto the SAM modified surface increases the contact angle to 102.40° indicating the enhancement in the hydrophobic nature of the surface due to the presence of long alkyl chain from the LPS moiety. In contrast, after attachment of the anti-leptospiral antibody to LPS anchored DT-modified surface through the antigen–antibody interaction a decrease in the contact angle of 90.00° is noted; revealing its switch over to a slight hydrophilic nature due to the presence of hydrophilic amino acids in the antibody. Thus these results provide evidence for the modification steps used in our study for the fabrication of sensor.

Fig. 1 displays the representative AFM images of gold electrodes before and after each modification step employed for the fabrication of biosensor. As seen from Fig. 1, the bare gold surface showed a smooth surface with an average height of 3.45 nm (Fig. 1A). After the formation of monolayer of DT, the surface height increased to 9 nm (Fig. 1B), further upon immobilization of LPS, the surface height increased to12.8 nm (Fig. 1C). Finally, after the functionalization with antibody, the surface height increased further up to 16 nm (Fig. 1D). The increasing surface height along with the roughness factor is mainly attributed to the adsorption of molecules in each and every modification step. These results clearly confirm the functionalization of Au electrodes with the target molecules and reaffirm the formation of different layers on Au surface during the modification.

3.2. Impedimetric response of the chemically modified gold electrodes

Before modification, electrochemical response of the bare electrode was recorded. Bare Au electrode shows a clear redox peak observed at 0.276 V and 0.201 V in cyclic voltammograms due to the redox reaction occurs on the surface of gold electrode. Initially, a self-assembled monolayer of DT is formed on the gold electrode, which leads to the formation of a highly organized hydrophobic monolayer that blocks the electron transfer process. A drastic decrease (2-fold in comparison to bare Au) in the redox current is noted in CV after the addition of DT, which indicates that the electron transfer reaction is inhibited (Fig. 2A). The Nyquist plot shows, an obvious semicircle up to low frequency with the R_{CT} value of 1.28×10^4 Ω cm^2, reveals the charge transfer controlled process for the redox probe on SAM modified electrode (Fig. 2B). In Fig. 2B inset shows the Nyquist plot of bare gold electrode shows a small semicircle with a straight line in the Nyquist plot of impedance spectroscopy and the corresponding R_{CT} value is measured to be 3850 Ω cm^2 which defines the characteristic of a diffusion-controlled process. This EIS data indicates the successful formation of DT monolayer on the gold electrode and hindering the electron transfer reaction on the surface which correlates well with the CV results. This is due to the inability of the redox species to penetrate the monolayers onto the conductive electrode surface. To examine the surface structure of DT monolayer modified electrode XPS studies were performed. **Fig. S3A** depicts the XPS survey spectrum of DT monolayer modified Au electrode and **Fig. S3B** reveals the presence of sulphur peaks (S2p) at 162.3 eV corresponding to the sulfhydryl group. DT can interact with Au electrode through



Scheme.1. Schematic representation of the biosensor construction process. (a) bare gold electrode, (b) DT monolayer modification, (c) DT + LPS modification, (d) DT + LPS + BSA addition and (e) DT + LPS + BSA + Anti-leptospiral antibody modification.

Table 2
Elements present in the equivalent circuit used for fitting the impedance data of proposed sensor (Grippotyphosa).

Monolayer	$R_S u$	CPE (x 10^{-7})	Freq	R_{CTCT}	Warburg(x 10^{-5})	Chsq
Bare	342	0.104	0.914	3964	7.793	7.43 x10^{-3}
SAM	444.7	3.649	0.861	1.669 x10^{5}	4.786	1.59 x10^{-2}
LPS	409.7	2.785	0.828	4.19 x10^{5}	2.279	1.48 x10^{-2}
BSA	622.7	6.01	0.835	6.86 x10^{5}	2.241	9.09 x10^{-3}
HS	357.8	3.149	0.818	3.24 x10^{6}	1.179	2.21 x10^{-2}
HES	353.9	6.572	0.814	2.218 x10^{6}	1.097	3.14 x10^{-2}
HC	376.2	3.67	0.807	1.757 x10^{6}	1.586	2.95 x10^{-2}

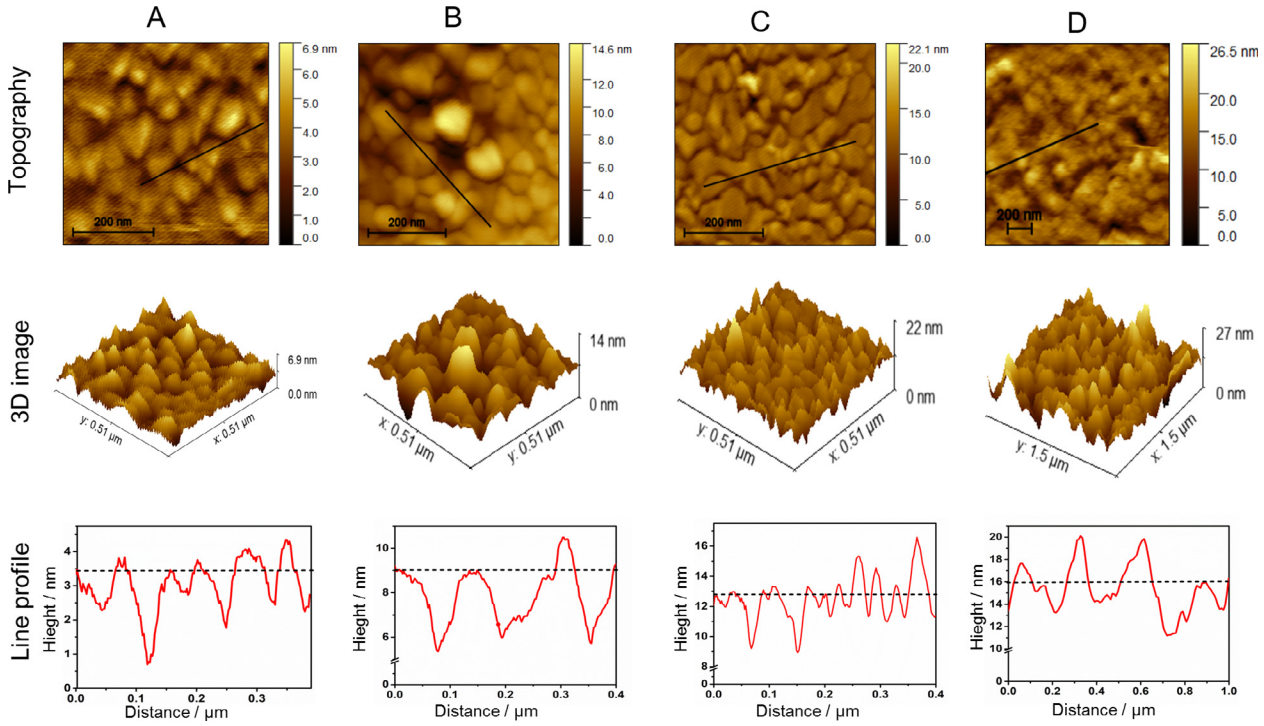


Fig. 1. Representative AFM images displaying surface topography, 3D-images and line profiles of various modified electrodes used in this study. Panel A- bare gold electrode, Panel B- DT-monolayer, Panel C- leptospiral LPS modified and Panel D- anti-leptospiral antibody functionalized electrodes respectively.

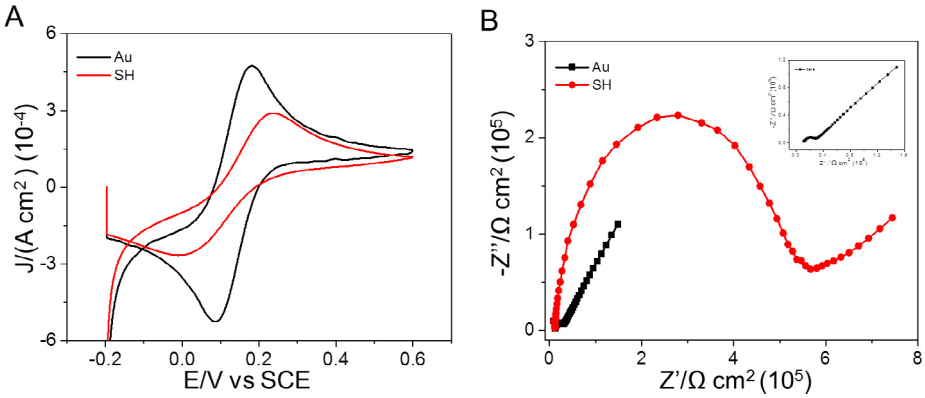


Fig. 2. Electrochemistry characterization of the sensing electrode. (A) Cyclic voltammograms, and (B) Impedance (Nyquist) plots in an aqueous solution containing 1 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M NaF for bare gold electrode and DT monolayer modified electrode compared to bare Au electrode. (Inset. Nyquist plot of bare Au electrode).

sulfhydryl groups forming Au-S interaction leading to the formation of organized SAM.

3.3. Functionalization of SAM coated sensing electrode

In order to achieve the serogroup detection of leptospirosis, the SAM coated electrode was functionalized with the addition of leptospiral LPS. Such chemical modification is carried out by anchoring the leptospiral LPS onto the DT monolayer films through hydrophobic interactions between the long aliphatic chains from LPS with the alkyl chains of the monolayer. To detect the optimal concentration of leptospiral LPS for the detection of leptospirosis the impedance responses were recorded for the addition of different concentrations of leptospiral LPS to the SAM coated surface in

the range from 10 pM, 100 pM, 500 pM, 100 nM, 500 nM to 1 μM and their corresponding R_{CT} values were measured to be 7.6×10^5 Ω cm^2, 9.74×10^5 Ω cm^2, 10.09×10^5 Ω cm^2, 11.76×10^5 Ω cm^2, 11.79×10^5 Ω cm^2 and 12.44×10^5 Ω cm^2 respectively (Fig. 3A). The Binding of LPS is identified to be remarkably effective in the concentration range of 100 nM (Fig. 3B). The increase in R_{CT} is probably related to both an increase in negative charges and thickness of the molecular layer on the electrode surface. However, in some serogroups the effect of mass transfer persists at the lower frequency range.

To further probe into the structure of modified surfaces, XPS analysis of SAM coated gold electrode was carried out. XPS spectra of these modified electrodes display peaks due to nitrogen and phosphorous that appear at 399.1 eV and 132.8 eV due to the presence of LPS apart from the carbon and sulphur peaks noted at 285 eV and 162.9 eV arising from the DT monolayer respectively **(Fig. S4)**. These results clearly suggest the anchoring of LPS onto a monolayer modified gold electrode. In order to saturate the electrode surface, BSA was immobilized and used as a surface covering agent. The addition of BSA, which blocks the surface other than to LPS has the R_{CT} value of 4.8×10^4 Ω cm^2 and allows the binding of antibodies specific to the target analyte.

3.4. Development of serovar specific biosensor

To develop the serovar-specific biosensor and to eliminate the possible cross reactive binding of other serogroups, independent experiments were conducted using LPS of different leptospiral serogroups. Moreover to improve the specific binding and to decrease the nonspecific binding from other sera, homologous sera confirmed by MAT and ELISA analyses were used for the study. The LPS modified electrode was immobilized with the anti-leptospiral antibodies, for that the laboratory-confirmed leptospirosis patient's serum samples were used. EIS response of the sensor is drastically increased further, which indicates strong affinity between the LPS and anti-leptospiral antibodies. In order to avoid hook effect of the antigen antibody interaction we design the experiments at different dilutions of the patient's serum containing the circulating anti-leptospiral antibodies against the leptospiral LPS. The representative R_{CT} values for the dilution of 1:100 (5.682×10^7 Ω cm^2), 1:200 (5.475×10^7 Ω cm^2), 1:500 (5.089×10^7 Ω cm^2) and 1:1000 (4.77×10^7 Ω cm^2) respectively were determined (**Fig. S5A and S5B**). These results indicate the decreasing R_{CT} value while increasing the dilution of the sera. Upon increasing the dilution of antibody further the R_{CT} values are found to be decreased, confirming the decreasing concentrations of antibody. For all further experiments, a dilution of 1:1000 was used. At the same time, upon addition of healthy control serum samples there was no significant increase in R_{CT} is noted indicating the selectivity of the biosensor against only leptospiral specific antibodies.

Since the focus of our study is the serogroup specific detection of leptospirosis, we analyzed the interaction between Grippotyphosa LPS molecule to the MAT and ELISA confirmed patients' sera (anti-leptospiral antibodies). Hence the sensing electrode consisting of Grippotyphosa LPS was subjected into various MAT confirmed leptospiral cases which have specific reactivity to Australis, Autumnalis, Ballum, Canicola, Pomona, Hebdomadis, Pyrogenes, Sejroe and Grippotyphosa. Their corresponding impedance responses were shown in Fig. 4A from which their R_{CT} values were determined and shown in Fig. 4B. Together the sensing electrode comprising of Grippotyphosa LPS has a higher affinity to the Hebdomadis ($3.6x\ 10^8$ Ω cm^2) and Ballum ($2.46x\ 10^8$ Ω cm^2) compared to the Grippotyphosa positive case (7.3×10^8 Ω cm^2). From these results, it is clear that the proposed biosensor possesses higher specificity to the homologous sera than the heterologous sera.

A limited number of clinical samples (n = 12) were used for the evaluation to assess the ability of biosensor in analyzing the 'real world' samples. As mentioned before the clinical samples were analyzed with serological assays such as MAT and ELISA to confirm the presence of anti-leptospiral antibodies prior to the biosensor experiments. The results summarized in Table 3 reveal that the sensor could able to differentiate the healthy individual sera. The representative histogram (Fig. 4C and 4D) demonstrates the specificity associated with each sensor. From these results, it is clear that the sensor has higher affinity to LPSs of Hebdomadis (8.694×10^7 Ω cm^2), Pyrogenes (2.071×10^7 Ω cm^2), Sejroe (1.331×10^7 Ω cm^2), Ballum (1.033×10^7 Ω cm^2) followed by Australis (0.979×10^7 Ω cm^2), Canicola (0.707×10^7 Ω cm^2), Autumnalis (0.683×10^7 Ω cm^2), Bataviae (0.547×10^7 Ω cm^2), Pomona (0.165×10^7 Ω cm^2) and Javanica (0.142×10^7 Ω cm^2) **[Fig. S6A-J]**. Then there is a slight increase in R_{CT} value upon addition of BSA, which indicates the prevention of nonspecific interactions on the LPS functionalized electrode. Further addition of specific anti-leptospiral antibody (leptospirosis confirmed (MAT) patients samples), a sharp increase in the R_{CT} was observed. The representative R_{CT} values for Hebdomadis, Pyrogenes, Sejroe, Ballum, Australis, Canicola, Autumnalis, Bataviae, Pomona and Javanica were 15.69, 4.252, 3.117, 2.502, 2.926, 1.343, 1.172, 2.402, 0.225 and 0.188×10^7 Ω cm^2 respectively. These findings reveal that the antigen –antibody complex was formed on the surface of sensing elec-

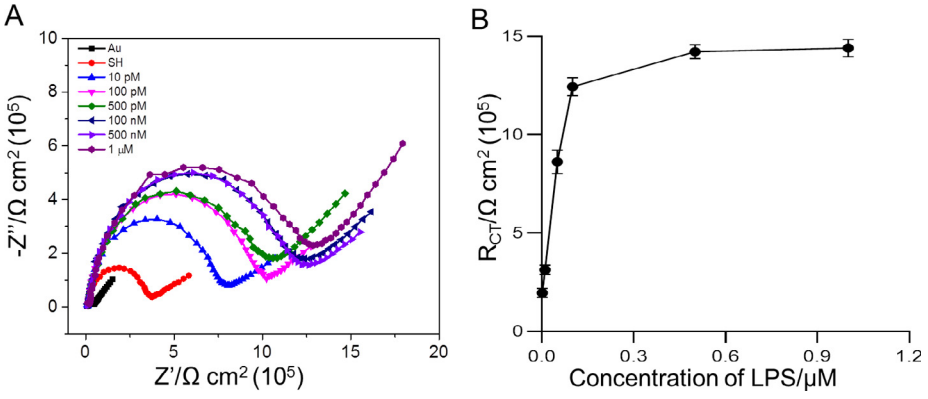
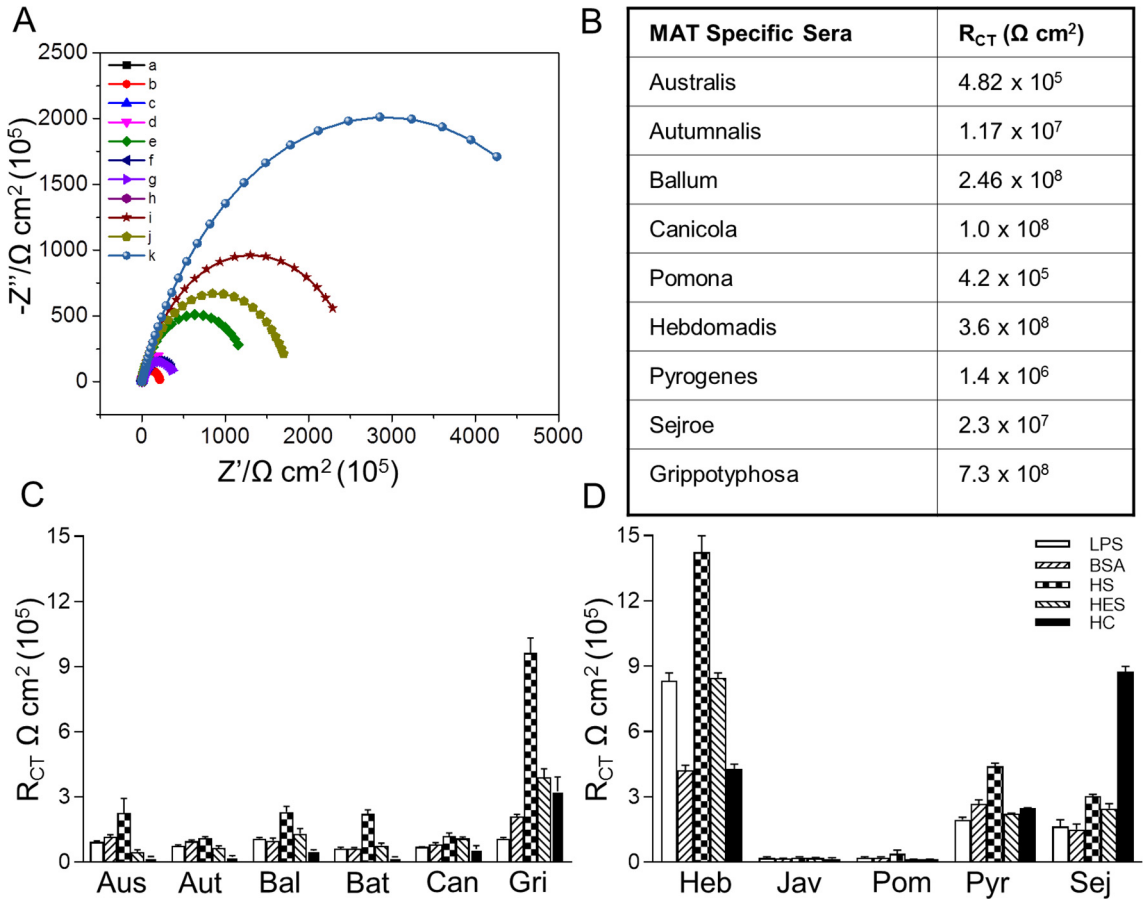


Fig. 3. Optimization of leptospiral LPS concentration. (A) Impedance plots in an aqueous solution containing 1 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M NaF for different concentrations of Grippotyphosa lipopolysaccharide (Au- Bare Au, SH- DT-monolayer, 10 nM – 1 μM- different concentrations of LPS). The lines show the fitting of measured impedance data points using Randle's equivalent circuit. (B) Plot of the ratio, R_{CT}/R^0_{CT}, (where R^0_{CT} is the resistance to the electron transfer reaction before LPS addition and R_{CT} is the measured values for each addition of LPS) vs. concentration of Grippotyphosa LPS.



MAT Specific Sera	R_{CT} (Ω cm^2)
Australis	4.82 x 10^5
Autumnalis	1.17 x 10^7
Ballum	2.46 x 10^8
Canicola	1.0 x 10^8
Pomona	4.2 x 10^5
Hebdomadis	3.6 x 10^8
Pyrogenes	1.4 x 10^6
Sejroe	2.3 x 10^7
Grippotyphosa	7.3 x 10^8

Fig. 4. Serovar specific analysis. (A and B) Impedance plots in an aqueous solution containing 1 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M NaF for the Grippotyphosa LPS against homologous and heterologous sera (a- bare Au, b- DT monolayer, c- DT + GrippoLPS, d- DT + Grippo LPS + BSA, e-j (DT + Grippo LPS + BSA + Heterologous sera, k- DT + GrippoLPS + BSA + homologous sera), and their R_{CT} values. (C and D) Histrogram of the R_{CT} values for different serovars namely Australis, Autumnalis, Ballum, Bataviae, Canicola, Hebdomadis, Javanica, Pomona, Pyrogenes and Sejroe respectively.

Table 3
R_{CT} values of the sensing electrode consisting of various leptospiral lipopolysaccharide with respect to the homologous and heterologous sera.

Serovar	R_{CT} values of different modified electrodes (x 10^7 Ω cm^2)				
	LPS	BSA	Homologous sera	Heterologous sera	Healthy sera
Australis	0.979	1.092	2.926	0.574	0.193
Autumnalis	0.683	0.893	1.172	0.745	0.156
Ballum	1.033	1.103	2.051	1.538	0.475
Bataviae	0.547	0.666	2.402	0.648	0.169
Canicola	0.707	0.710	1.343	1.158	0.442
Grippotyphosa	1.134	2.199	10.32	4.290	3.920
Hebdomadis	8.694	4.459	15.69	8.694	4.093
Javanica	0.142	0.151	0.188	0.169	0.109
Pomona	0.165	0.177	0.225	0.109	0.128
Pyrogenes	2.071	2.874	4.252	2.230	2.452
Sejroe	1.331	1.251	3.117	2.698	8.991

trode. Further the binding of anti-leptospiral antibody to the specific leptospiral LPS to form the antigen–antibody complex occur on the gold electrode as confirmed by XPS, which shows S 2p peak at 162.5 eV, and N 1 s peak at 400.1 eV. Moreover, their corresponding intensity values were increased when compared to SAM and LPS modified electrodes confirming the presence of antibody (**Fig. S7**) and reconfirms the binding.

3.5. Selectivity of the developed biosensor

In order to investigate the selectivity of this particular biosensor, the modified electrode has been tested with sera samples of dengue and typhoid patients, where the R_{CT} values were measured to be 2.3 × 10^6 Ω cm^2 and 1.4 × 10^6 Ω cm^2 and compared the R_{CT} value of *Leptospira* specific sera of 7.3 × 10^8 Ω cm^2, which was significantly higher. As shown in the Nyquist plot (Fig. 5A and 5B), the biosensor responses to the other samples are significantly lower due to the nonspecific interaction with the antibody, which makes the sensor electrode very specific to the target analyte of leptospiral LPS. Further, we also performed the electrochemical response for non-pathogenic leptospiral LPS to the anti- leptospiral antibody (patient's serum) **Fig. S8A**, where the R_{CT} value was found to be lower (2.8 × 10^6 Ω cm^2) compared to the pathogenic LPS. Also the healthy control sera showed decreased R_{CT} (2.0 × 10^6 Ω cm^2) **Fig. S8B**, thus confirming the higher specificity of the proposed biosensor.

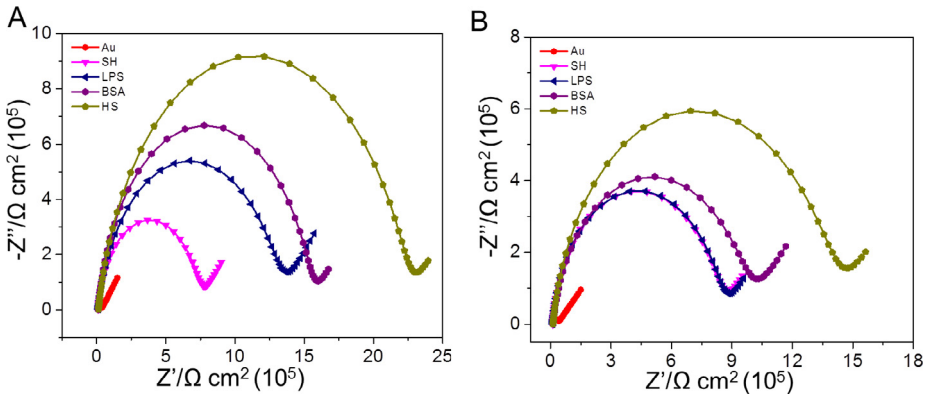


Fig. 5. Selectivity of the proposed sensor. Nyquist plots for the leptospiral LPS reactivity against the dengue (A) and typhoid (B) patients sera. Legends: (Au- bare Au, SH- DT - monolayer, LPS- DT + LPS, BSA- DT + LPS + BSA complex, HS- DT + LPS + BSA + Patient's sera (Dengue or Typhiod).

We also compared our sensor performance with that of other methods or functionalized materials used for LPS detection (Table 4). Though previous detection methods mostly used *E.coli* LPS [27,28] and this is the first attempt on leptospiral LPS based detection for real-world sample diagnosis with a low limit of detection range consistent with other LPS detection methods.

4. Discussion

In the outer membrane of leptospires, LPS is the major antigenic component present and it is thought as the best immunogenic molecule because they are serogroup specific [29]. Since the laboratory diagnosis for leptospirosis mainly depends upon the serological techniques, and the serogroup-specific MAT is the most widely used reference method these days. Generally, MAT detects both anti-leptospiral, IgM and IgG and most of the agglutinating antibodies confronted in MAT are IgM that is produced against the LPS in the acute phase of illness [30,31]. Even though MAT is considered as the gold standard test for the diagnosis of leptospirosis, but it is complex to control, perform, and interpret. Mostly it is performed with locally circulating predominant leptospiral serovars as live antigens and MAT forms the bases for serological diagnosis and for the classification of leptospires. Especially during outbreak condition, investigation of such a large number of serum samples by a complicated technique like MAT may compromise the quality of results. Apart from these drawbacks, MAT also suffers from other major issues like cross-contamination of the antigen cultures, interference of the culture medium to affect the MAT titers, and the maintenance of a large number of strains are hazardous for laboratory workers [32].

Under critical circumstances, the use of an alternate rapid serological diagnostic test is highly warranted. Serological tests like the microcapsule agglutination test (MCAT), Lepto Dipstick, Lepto Lateral Flow, and Lepto Dri Dot have been evaluated as rapid tests for leptospirosis as alternative techniques. But the majority of them are genus-specific and they mainly suffer from low sensitivity and specificity during the acute stage of the disease [33–36]. An attempt was made to develop a serogroup specific diagnosis of leptospirosis by ICG-LFA, but in this array the Ballum LPS showed cross-reactivity with other serovars [16].

Further to improvise the rapidity of the technique and make it applicable in outbreak situations, a point of care serogroup specific diagnosis, LPS based electrochemical biosensor is proposed and demonstrated in this work for the first time and evaluated for the diagnosis of leptospirosis. This particular sensor is fabricated by immobilizing LPS onto the DT modified gold electrode. This test could potentially be assessed and employed as a point of care alternative test for MAT to detect the locally prevalent infecting serogroups in endemic regions and offers many advantages.

5. Conclusions

In conclusion, our results clearly demonstrate the rapid diagnostic method for the specific diagnosis of acute leptospirosis using electrochemical impedance measurements by employing LPS based serovar detection strategy. The proposed biosensor could sense the wider concentration of leptospiral LPS ranging from 10 pM to 1 μM. EIS measurements of LPS based sensor offers a simple and promising method to develop extremely effective and sensitive serogroup specific diagnosis of acute leptospirosis. This method has several significant advantages towards the prevention of leptospirosis diseases, whereby this specific sensor could be directly employed to identify the serogroup specific leptospiral agglutinins. Further, the proposed sensor could also help for the timely initiation of antibiotics that may be critical for the treatment of leptospirosis.

Table 4
Comparison of selected methods for detection of LPS.

Recognition material	Detection Method	Detection range	Sensitivity	Reference
293/hTLR4-MD2-CD14-pGL4.26-m.cherry-NF-kB cells	Fluorescent	0.01 – 100 ng mL^{-1}	0.01 ng mL^{-1}	[37]
Aptamer	Electrochemical Impedance Spectroscopy	0.001 – 1.0 ng mL^{-1}	0.001 ng mL^{-1}	[38]
Polymyxin B	Electrochemical Impedance Spectroscopy	0.2 – 0.8 ng mL^{-1}	0.2 ng mL^{-1}	[39]
Au NPs/ CramoLLlectin	Electrochemical Impedance Spectroscopy	Not reported	200 μg mL^{-1}	[40]
Aptamer	Electrochemical Impedance Spectroscopy	0.01 aM – 1.0 pM mL^{-1}	7.94×10^{-21} M mL^{-1}	[41]
rhTLR4/MD-2complex	Differential Pulse Voltammetry	0.0005 – 5 EU mL^{-1}	0.0002 EU mL^{-1}	[42]
Dodecanethiol- modified Au electrode	Cyclic Voltammetry and Electrochemical Impedance Spectroscopy	10 pM – 1 μM mL^{-1}	10 pM mL^{-1}	This study

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2021.108005.

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