Electrochemical study of biophysical interaction of *Brucella abortus* antigen

[1]Ajay Kumar Gupta, [2]V. K. Rao, [3]D.T Selvam

Scientists
Centre of Excellence for Advanced Materials, Manufacturing, Processing and Characterization,
Vignan's Foundation for Science Technology & Research University, Guntur-522213, India

Abstract-Study of biophysical interactions have been carried out using specific combination of proteins such as *Brucella abortus* cell envelop protein (as antigen) and its complementary antibody (raised in mice and rabbit). MWCNT Paste electrodes (MWCNTPEs) were used in this study. Linear range was found in the range of 2.5 µg/mL–100 µg/mL. The detection limit was found 1 ng/mL. By ELISA it is possible to detect 50 µg/mL only. Experiments were conducted using 110 suspected patient serum samples and results were compared with ELISA and Rose Bengal Plate test (RBPT). The immunosensor was found to be highly sensitive compared to ELISA and RBPT. We also studied the shelf life of prepared immunosensor. The shelf life of immunosensor was found to be 44 days.

***Index Terms*- Immunosensor; ELISA; MWCNT-paste electrode; CE-protein antigen.**

***Abbreviations:* MWCNTs, Multiwalled carbon nanotube; MWCNTPE, Multiwalled carbon nanotube paste electrode; CE-protein antigen, Cell envelope protein antigen; ELISA, enzyme linked immunosorbent assay; RBPT, Rose Bengal Plate test; PCR, Polymerase chain reaction; CAb, capturing antibody; RAb, revealing antibody; ALP, alkaline phosphatase; PBS, phosphate buffer saline; DEA, Diethanolamine; LPS, lipopolysaccharide; CPE, Carbon paste electrode; CV, Cyclic Voltammetry.**

I. INTRODUCTION

Over the past decade, the interest in the development of a simple, inexpensive and disposable biosensor for the detection of pathogens under field conditions has increased. Recently, extensive work has been done on the use of electrochemical immunosensors for environmental [1] and clinical diagnosis [2,3]. Electrochemical immunosensors combine the high specificity of traditional immunochemical methods with the low detection limit of a modern electrochemical system. The electrochemical techniques employed for immunoassays are mostly based on amperometric methods [2-4] and voltammetric methods [5]. For immunosensors based on electrochemical methods, it is also possible to miniaturize the electrode for field applications [4]. Various types of electrodes such as carbon paste electrode (CPE) [3,6], glassy carbon electrode [5], platinum electrode [7] and gold electrode [8] have been used for electrochemical immunosensors.

Brucella abortus is gram negative and intracellular micro-organism. It causes brucellosis disease which is zoonotic in nature, and hence, it can be transferred from cattle to a human and remains pathogenic. The bacteria are transmitted by contact with infected animal, ingestion of infected milk, milk products and inhalation of aerosols. This disease rarely transmitted from mother to child in case of human. Brucellosis is considered by FAO, WHO and O I E as the most widespread zoonosis in the world. The prevalence of this disease is very high in India [26].

Cell Envelope (CE) protein is an outer membrane protein of *B. abortus* and the molecular weight of immunogenic CE-protein antigen varies from 11.8 kDa to 110.8 kDa [**27**,**28**,29]. Several standard tests such as standard plate agglutination test, standard tube agglutination test, acidified plate antigen test, rivanol precipitation plate antigen test, serial dilution milk ring test, complement fixation test, standard buffered brucella antigen card test, mercaptoethanol agglutination test and ELISA have been used in the diagnosis of brucellosis. [30]. These tests are mainly based on the detection of antibodies directed against the lipopolysaccharide portion of the cell membranes. Tests are also available for the detection of *Brucella* antigen. Al-Shamahy et al. reported enzyme linked immunosorbent assay for *Brucella* antigen detection in human sera [31]. Al-Farwachi et al. also reported modified ELISA test for detection of *Brucella* antigen in the aborted ovine fetal stomach content [32]. Immuno-histochemical technique and polymerase chain reaction (PCR) were also carried out for detection of *Brucella* infection [33,34]. Several methods were reported in the literature for detection of brucellosis in recent years. However, these methods are either time-consuming and/or of low in sensitivity. Some of the methods require highly qualified personnel (e.g., PCR) or sophisticated instrumentation (PCR, florescence microscopy, and flow cytometry) [35-37]. The requirement of highly qualified manpower and sophisticated instrumentation make them less useful in the diagnosis.

Recently, MWCNT have come to the fore front of electrochemical research due to its attractive electronic, chemical and mechanical properties [9-11]. The tubular form of MWCNT has got appreciable electronic properties which suggest that they might mediate the electron transfer reactions with an electroactive species in solutions [12,13]. MWCNTs can also be pretreated with concentrated acids in order to open the nanotubes and create carboxylic groups and thus exhibit an intense catalytic activity towards the electrochemical oxidation of phenolic derivatives [14]. MWCNTPEs were recently utilized for biosensor applications [15]. Screen printed electrodes (SPEs) and its modification with MWCNTs and gold nanoparticles were also used for bio-sensing and immunosensing applications [16-20]. Carbon nanotubes (CNTs) were also used in immunosensing by using transducers such

as field effect transistors [21]. In one of the reports, antibodies were attached to CNTs using covalent and non-covalent approach [22]. Recently, MWCNTPE was prepared using ionic liquid and was reported for bio-sensing applications [23]. The CNT paste electrodes (CNTPEs) without any support were used for biosensing applications [24,25]. In this present paper, we used MWCNT-paste electrode for detection of CE protein antigen. Although MWCNT-paste electrode is reported in the literature however, in this study, a unique detection approach is carried out. And this is the reason which make this present study unique form other study (carried out using MWCNT-paste) available in the literature. In this study, we selected a unique biocompatible porous nanomaterial paste (MWCNT-paste) for electrochemical study. However, only the selection of nanomaterial-paste is not enough. In order to make the unique immunosensor and to achieve maximum sensitivity and very good shelf life, it was mandatory to optimize the composition of MWCNTs and paraffin oil in MWCNT-paste. We selected that composition of MWCNTs and paraffin oil which was having maximum electrochemical response and minimum potential barrier [40]. It was very difficult to find maximum shelf life and sensitivity of immunosensor without previous step. The prepared MWCNT-paste was porous in nature and therefore, was very helpful to trap the bio-molecules in their crevices and voids. Thus MWCNT-paste was helpful to provide the biocompatible microenvironment to the bio-molecule and hence, resulted in to achieve good shelf life of immunosensor. In this study, we used anti-*B. abortus* CE-protein antibody raised in mice (as capture antibody) and anti-*B. abortus* CE-protein antibody raised in rabbit and conjugated to alkaline phosphatase (ALP) (as revealing antibody) for the detection of *B. abortus* CE-protein (antigen) based on sandwich ELISA system. The capture antibodies were adsorbed (physical adsorption) on MWCNT paste electrode, which were subjected to sequential incubation in CE-protein (antigen) and revealing antibody. In addition to this, we compared the *B. abortus* CE-protein amperometric immunosensor with conventional methods used in serodiagosis of brucellosis like plate ELISA [38] and RBPT [39]. By using electrochemical methods, cost-effective and field-deployable diagnostic devices can be made. In addition to amperometry and CV, various studies such as EIS, Raman, UV-VIS and surface area analysis were also carried out in this present study.

In this present study we also observed post-zone phenomenon, cause of false negative reaction. For the detection of antigen-antibody complex formation, the ratio between antigen and antibody is the influencing factor. When antigens and antibodies are in optimum ratio, lead to cross linkage and hence agglutination appears, resulting in positive reaction. But when antigen concentration become in excess (post-zone phenomenon), agglutination is hidden by mass of unagglutinated antigens and this resulted in false negative reaction. This phenomenon is known as post-zone phenomenon. These false-negative reactions can be detected by higher dilutions of antigen sample, which reduces the antigen concentration into the range that produces visible agglutination. Hence, it is necessary to dilute the sample to confirm whether it is negative or not. In this present study, we observed post-zone like phenomena with electrochemical immunosensor. However, no false negative result was observed. Also the sensitivity of immunosensor was much higher than ELISA.

II. MATERIALS AND METHODS

Apparatus

Cyclic Voltammetry (CV) experiments and chrono amperometric experiments were performed with CH Instrument, 440A. The detection was carried out in a 10 mL electrochemical cell, with MWCNTPE as a working electrode. An Ag/AgCl/Satd. KCl was used as reference electrode and platinum wire was used as counter electrode. Nano-spectrophotometer (Implen) has been used for finding change in absorbance. Sonication has been done by using Sonics & Materials Inc.. SEM (Quanta 400ESEM The Netherlands) has been used for scanning electron micrograph (SEM) studies. Raman study was carried out by using Renishaw Invia Raman Microscopy and surface area was calculated by using Micromeritics, USA, ASAP 2020 instrument.

Reagents and chemicals

The detection buffer consisted of 0.1M diethanolamine (DEA) containing 0.02M of magnesium chloride and 0.1M sodium chloride (NaCl) with an adjusted pH of 9.8. The antigen/antibody buffer consisted of 0.02M phosphate buffer with 150 mM NaCl (PBS pH 7.4). The washing buffer consisted of 100 mM of Tris and 100 mM NaCl and adjusted to pH 7.2. It has been reported that pH 7.4 was best optimized for the antibody immobilization [41] and the same was used in our experiments. This buffer was made with 0.02M of PBS containing 0.03% bovine serum albumin (BSA) and its pH was maintained at 7.4. Standard solution of the CE-protein antigen was prepared (0.5 mg/mL) in PBS of pH 7.4 and the required dilutions were made as and when required. Anti-*B. abortus* CE-protein antibody raised in mice (CAb) and anti-*B. abortus* CE-protein antibody raised in rabbit and conjugated to ALP were diluted using 100 mM Tris containing 100 mM NaCl with a pH 7.4. The dilutions were prepared freshly before use. In spectroscopic study, para-nitro phenyl phosphate (PNPP) was used as a substrate of ALP. Bovine serum albumin (BSA), Diethanolamine (DEA), 1-napthyl phosphate monosodium salt, p-nitro phenyl phosphate (PNPP), Tris–HCl, PBS buffer were purchased from Sigma Chemicals and all other chemicals used were of analytical grade. Capture antibody (CAb) and revealing antibody (RAb) were raised in our laboratory. The anti-CE-protein antibody raised in rabbit was tagged with ALP conjugate as per standard protocols in our laboratory.

Preparation of Multiwalled Carbon nanotube Paste Electrode (MWCNTPE)

Prior to preparation of MWCNT paste, 0.05g of MWCNTs were treated with 60 mL of 2.2M nitric acid for 24h in stirring condition and then it was sonicated for 30 min with the help of ultrasonicator. Further it is washed with distilled water to remove the acid. Later, it was dried for 1h in nitrogen atmosphere and finally MWCNTs were kept at 110^{o}C for 3h to remove moisture. Multiwalled carbon nanotube paste was prepared in the optimized ratio of 65:35 as per our previously reported method [39]. Further prepared MWCNTs paste was filled in an electrode holder, made up of teflon cylinder (4mm inner diameter). To obtain tight packing, MWCNTs paste was filled in layer by layer fashion inside the cavity of teflon holder. The filled MWCNTs paste

can be pushed toward the electrode surface as and when required with the help of screw type of arrangement, possessed by the teflon cylinder. To ensure the electrical connection, conductivity between brass screw and the tip of MWCNTs paste was checked with the help of a multimeter. In order to improve its electrochemical characteristics, MWCNTPE was conditioned in DEA buffer solution of pH 9.8 for 3 min at a potential of 1.7V. Later the capture antibodies were immobilized on these electrodes. The image of components of teflon electrode and the image of teflon electrode are shown in Fig. 1 (a) and Fig. 1(b).

CE-protein preparation

B. abortus S99 bacterial strain regularly maintained in the laboratory in 20% glycerol stock was revived in 5 mL BHI broth at 37°C for overnight. 3 mL of overnight grown culture was inoculated into 300 mL of BHI broth and incubated in an incubator shaker at 37°C with constant shaking (180 rpm). Bacterial cells were then harvested after inactivating the cells by formaldehyde treatment for one hour. The bacterial pellet obtained was washed twice with sterile PBS and resuspended into 100 mL of buffer 1, pH 8.0 (Tris–HCl 15 mM, Sucrose 0.45 mM, EDTA 8 mM, lysozyme 0.4 mg/mL). The bacterial suspension was incubated in water bath at 47°C for 15 min and then centrifuged at 10000 rpm for 15 min. Pellets were resuspended in 10-15 mL buffer 2, pH 7.6 (Tris–HCl 50 mM, $MgCl_2$ 5 mM, PMSF 2 mM). Further bacterial suspension was sonicated by using Vibrocell (Sonics) sonicator. The bacterial suspension was placed in crushed ice to prevent degradation of protein and 5 cycles of 5 min each with a pulse after every 8 sec and amplitude at 40W was given for the complete sonication of bacterial cells. The sonicated suspension was centrifuged at 6000 rpm for 30 min at 4°C. Supernatant was collected and subjected to ultra centrifugation at 43500 rpm at 4°C for 90 min (Sorvall ultra pro). The obtained pellets were then resuspended finally in 1.5 mL of buffer 3 of pH 7.6 (Tris–HCl 50mM, PMSF 2mM), and again centrifuged at 10000 rpm for 10 min and the supernatant was stored at -20°C. The protein concentration was estimated by Lowry *et al.* [42] and found to be 0.5 mg/mL. In addition to this the protein was also characterized by SDS-PAGE [29].

Raising of Antibody

Polyclonal antibodies against CE-protein antigen were raised in rabbit and mice. First dose of CE-protein was given at a concentration of 20-30 µg per mice and 500 mg/rabbit with Frund's complete adjuvant (Difco). Four booster doses were then given at an interval of 7 days with incomplete Frund's adjuvant (Difco). The animals were regularly bled and serum was collected after 7 days to check the antibody titre by plate ELISA. The animals were finally bled and serum was pooled with a titre of 1:51,200 in mice and1:1,20,000 in rabbit. The serum containing polyclonal antibodies against CE-protein antigen was stored at -20°C in aliquots for further use. The rabbit sera was further subjected to IgG purification and purified IgG was conjugated to alkaline phosphatase by using standard procedure. This polyclonal antibody raised in mice was used as capture antibody and the alkaline phosphatase tagged rabbit polyclonal antibody was used as revealing antibody in immunosensor. The generation of antibodies in animals used in this study has been approved by institutional animal ethics committee.

Steps in immunosensing of CE-protein antigen

A sandwich ELISA method as depicted in Schematic diagram (graphical abstract) was used. A known quantity (10 µL) of CAb of CE-protein antigen in PBS buffer was physically adsorbed on the MWCNTPE. It was left at 37°C for 1h and then washed. Subsequently it was blocked with buffered solution of 3% BSA for 30 min. This will reduce the nonspecific adsorption effect. Later the electrodes were incubated with the various concentrations of CE-protein antigen in PBS solution for 15 min. This results in selective antigen - antibody interaction. Electrodes were further incubated with revealing antibody for 15 min. The secondary antibodies (revealing antibody) form a complex with the secondary sites forming a sandwich ELISA assay [43]. After each step the electrodes were washed with Tris buffer containing NaCl at a pH 7.2 to remove any unbound antigen or antibody. The electrode was finally dipped in an electrochemical cell containing DEA buffer (pH 9.8) and a potential of 0.45V with respect to reference electrode (Ag/AgCl/Satd.KCl) was applied. After allowing 200s for the stabilization of the electrode to achieve constant current, the substrate 1-napthyl phosphate was added and the resulting amperometric current was noted. The output current obtained can be co-related to the concentration of CE-protein.

III. RESULTS AND DISCUSSION

Physical characterization of MWCNT paste

SEM was used for examining the morphology of MWCNT paste. In the form of paste, MWCNTs have a lot of voids and crevices, which are helpful in trapping the antibodies during the process of physical adsorption. SEM images were reported in our previous report [40].

Surface area, pore size and pore volume study

Surface Area analysis

In as prepared MWCNT paste, the single point surface area at p/p^o of 0.31 was 36.26 m^2/g. BET surface area was found to be 39.77±0.70 m^2/g whereas Langmuir surface area was 62.66±3.57 m^2/g. In the surface area analysis, BJH adsorption cumulative surface area of pores between 1.7 nm and 300 nm widths was 36.10 m^2/g whereas BJH desorption cumulative surface area of pores between 1.7 nm and 300 nm widths was 45.47 m^2/g.

Pore Volume and Pore Size analysis

In the pore volume analysis of as prepared MWCNT paste, single point adsorption total pore volume of pores at width of less than 108.77 nm and p/p° of equal to 0.98 was 0.16 cm^3/g. BJH adsorption cumulative volume of pores between 1.7 nm and 300

nm width was found to be 0.155 cm^3/g whereas BJH desorption cumulative volume of pores between 1.7 nm and 300 nm width was 0.16 cm^3/g.

In pore size analysis of as prepared MWCNT paste, BET adsorption average pore width (4V/A) was found to be 16.46 nm whereas BJH adsorption average pore width (4V/A) was found to be 17.15 nm and BJH desorption average pore width (4V/A) was found to be 14.06 nm. In addition to above studies, some more studies were also carried out and given as below.

Horvath-Kawazoe maximum pore volume at p/p° of 0.10 was 0.01 cm^3/g and median pore width was found to be 1.24 nm. Dubinin-Astakhov micropore surface area was 30.37 m^2/g and limiting micropore volume was found to be 0.02 cm^3/g.

From these observations, it can be concluded that this material has nanopores, which can help in trapping the biomolecules.

Electrochemical characterization of MWCNTPE

MWCNT Paste electrode was characterized for their electrochemical property towards 1mM K_3FeCN_6 (0.1M KCl) and 1mM 1-naphthol by CV. The details of the hydrodynamic conditions for getting maximum current and the potential for oxidation of 1-naphthol were reported elsewhere [44]. The speed of rotation of the stirrer has been fixed at 700 rpm [45] and the applied potential was fixed at 0.45V with respect to reference electrode. Figure 2 shows the CV of MWCNT-paste electrode in 1mM K_3FeCN_6 (0.1M KCl) in PBS.

Relative adsorption efficiency of MWCNT paste electrode and bare SPE

In order to investigate the relative adsorption efficiency of MWCNT paste electrode and bare SPE, spectroscopic experiments were carried out and presented. The RAb containing ALP conjugate (1:100) was adsorbed on these electrodes for 15min, and then washed with washing buffer (TRIS buffer pH 7.2 was used). Electrodes were exposed to 2 mL of 4mM para-nitro phenyl phosphate (PNPP). PNPP is a substrate for ALP. Optical density (O.D.) of this solution was noted at various intervals of time and the resulting plot was shown in Fig. 3. The O.D of PNPP solution was high for MWCNT paste electrode compared to that of bare SPE. This observation can be attributed to high RAb adsorption efficiency of MWCNT paste. Higher the adsorption of RAb, higher will be the sensitivity. The higher adsorption efficiency of MWCNTPE was due to presence of voids crevices which can act as traps for the antibodies and also due to interaction between protein and MWCNT's sidewall which can be ascribed to hydrophobic interactions between exterior fullerene surface and regions of high hydrophobic residue density within protein tertiary structure [45].

Raman study

The main feature in Raman spectra of CNT is first order band, which is disorder induced D band. In Raman spectra of MWCNTs (Fig. 4), this D band was appeared at about 1318 cm^{-1}. The tangential mode G band in Raman spectra of acid treated MWCNTs was appeared at about 1578 cm^{-1}, which was corresponding to the stretching mode in the graphite plane. The second order G band was appeared at about 2619 cm^{-1}. In this Raman spectrum of acid treated MWCNT there was no Radial Breathing Mode (RBM) because this Raman feature was associated with the small diameter inner tube. In case of large diameter tube, RBM signal is usually too weak to be observable and the ensemble average of inner tube diameter broadens the signal [46].

Electrochemical Impedance spectroscopic study

To confirm the antibody immobilization on the surface of MWCNT-paste electrode, electrochemical impedance spectroscopy (EIS) was carried out. EIS was employed to characterize the interface properties of the modified electrodes (in our case antibody immobilized MWCNT paste electrode). In a typical Nyquist plot, the semicircle portion corresponds to the electron-transfer resistance (Ret) at higher frequency range while a linear part at lower frequency range represents the diffusion limited process. In this report, EIS study of the electrodes were carried out in 1.0mM $Fe(CN)_6^{3-/4-}$ containing 0.1M KCl solution with a frequency range of 0.01Hz–10 kHz. As shown in Fig. 5 curve 'a' represents the spectra of unmodified MWCNTPE. After immobilization of antibody, the semicircle domain of Nyquist plot was increased (curve 'b') suggesting that antibody layer blocked the redox probe to diffuse toward the electrode surface. Hence, it confirms that antibodies were immobilized on the surface of MWCNT-paste electrode.

Optimization of revealing antibody (RAb)

Figure 6 gives the response of the porous MWCNTPE after blocking with BSA for 30 min and incubating the electrodes with RAb for 15 min. Various dilutions of RAb were incubated and tested for obtaining the blank current. There was an appreciable increase in background current when a dilution of 1:10,000 to 1:100 was used. This background current was due to nonspecific adsorption of RAb on the sensing surface. A dilution of 1:5,000 of the RAb was optimized for further studies.

Optimization of capture antibody (CAb)

For this optimization all the steps except the step of incubation of the antigen were performed. There was no nonspecific interaction (CAb–RAb interaction) at 1:10 concentration and the amperometric current was almost matched with the blank current obtained with 1:5,000 dilution of RAb. Thus, 1:10 concentration of CAb was optimized for detection.

Detection of CE-protein antigen

The MWCNTPE was initially incubated with CAb at optimized dilution for 60 min. Subsequently, that was blocked with 3% BSA for 30 min followed by incubation in CE-protein antigen. After incubation in CE-protein antigen, these electrodes were incubated with RAb for 15 min. Each step was followed by washing with Tris buffer (washing buffer) to remove any unbound antigen or antibodies. Now these electrodes were dipped in an electrolyte containing DEA buffer at pH 9.8. A potential of 0.45 V

vs. Ag/AgCl/Sat.KCl was applied and the amperometric current was measured. When the current reached a steady state value, the substrate was added and the rise in the current was noted. The amperometric response was found to be linear in the range of 2.5 µg/mL to100 µg/mL (Fig. 7a)**.** Detection limit was found to be 1 ng/mL (Fig. 7b). Detection limit was determined on the basis of the equation **[baseline current (blank current) + 3 X standard deviation (S.D.)].** Calibration plots were made in two figures (Fig. 7a & 7b) due to observance of post-zone phenomenon in this study. The value of R was different in both calibration figures because at low concentrations, variation found in the results are more that that found at higher concentrations. Calibration plots (Fig. 7a & 7b) were the results of mean values of 3 times replicates for each concentration of antigen. Here the blank current was 24 nA and standard deviation was 7.1%. Hence, according to above equation, any amperometric response above than 45.3 nA (24 nA +21.3 nA) was considered as confirmation of presence of antigen. The reproducibility of the immunosensor was evaluated from its amperometric responses to 50 ng/mL concentration of antigen and the relative standard deviation was found to be 7.1%. It may be noted in the Fig. 7 (b) that at low concentrations, the immunosensor has high response. This can be attributed to post-zone like effect. The post-zone like effect was also observed in detection of Hepatitis B antigen, *Francisella tularensis* antigen [47,48]. The false negative results of agglutination test were attributed to this phenomenon. The immunosensor does not give false negative results even at high concentration of the antigen. Hence, no need to dilute the samples repeatedly to remove the post-zone effect. The high sensitivity of MWCNT paste electrode can be attributed to good electrochemical behavior and high adsorption efficiency. We successfully tested this immunosensor with 110 suspected patient serum samples.

Patient Serum sample detection

110 suspected patient serum samples were tested amperometrically by using MWCNTPE and results are compared with ELISA and RBPT test results. Samples used for the electrochemical immunosensor, ELISA and RBPT were same. Furthermore, the patient samples were diluted 10 times and the results were presented in **TABLE 1.** It was found that even after diluting the samples 10 times, the immunosensor was able to detect, while ELISA & RBPT were unable to detect the diluted samples. These results showed that the sensitivity of electrochemical immunosensor was much better than ELISA and RBPT test. Criteria of the judgement about posiive or negative for electrochemical immunosensor were determined on the basis of the equation **[baseline current (blank current) + 3 X standard deviation (S.D.)].** Baseline current was found 24nA and standard deviation was found 7.1%. In ELISA, positive and negative was explained on the basis of change in O.D. (optical density). While in case of RBPT, positive and negative was explained on the basis of agglutination. A complete agglutination with equal volume of antigen was determined as positive result for RBPT.

In table 1, there were also some unclear samples. Unclear samples were those samples which have the response very close to the value obtained from the equation **[baseline current (blank current) + 3 X standard deviation (S.D.)].** Thus it is very difficult to determine these samples as positive or negative and hence, kept these samples in a separate category of **Unclear samples**. It may be noted that these unclear samples were found negative in case of ELISA and RBPT even after dilution by 10 times.

In this study, we observed post-zone phenomenon which may be the one of the most important reason of this considerable difference in the no. of positive samples obtained from electrochemical techniques and other techniques such as RBPT and ELISA. Due to post-zone phenomenon, there is a high probability of occurrence of false negative results. And this may be the reason that in this study, we observed a large number of negative samples in RBPT and ELISA test. These biological techniques such as RBPT and ELISA are based on agglutination reaction between antigen and antibody. A complete agglutination with equal volume of antigen was determined as positive result. This agglutination reaction is hidden by mass of unagglutinated antigens in case of post-zone phenomenon (observed in this study) and thus resulted in false negative reaction.

Thermal stability study of immunosensor

The shelf life of immunosensor is one important aspect of immunosensor. Hence, thermal stability of immunosensor was also studied at various temperatures (4^0C, 37^0C and 50^0C). For this study, initially capture antibody was immobilised on the electrode surface for 1h and then blocked with 3%BSA. Later these electrodes were kept at 4^0C for various durations. In the same manner, immunosensors were again prepared and kept at 37^0C and 50^0C for various durations. Prior to experiments, electrodes were further immobilized with antigen (25 ng/mL) for 15min and then with optimized concentration of RAb for 15min. Activity of immunosensor becomes lost at 50^0C within 2 days and in case of immunosensor at 37^0C, activity become lost within 7 days. But when we kept immunosensor at 4^0C and analyzed after certain different durations (4 days, 8 days, 12 days etc.), sensitivity of immunosensor was found to be more than 90% of their original response (first day response) even after 28 days. In another study, we prepared the PBS buffer solution (antigen and CAb dilution solution) containing 0.1% sodium azide, an additive to prevent fungal growth. And we found that sensitivity of immunosensor remained more than 90% of the original response (first day response) after 44 days. Thus, the immunosensor has a shelf life of 44 days.

Thus, in the form of paste, MWCNTs have a number of voids and crevices, which were helpful in trapping the antibodies during the process of physical adsorption as a result of which sensitivity increased several times. In this study, no false negative results were obtained by immunosensor and these results were concluded by repeating the experiments on diluted samples. Thus the immunosensor can be used for diagnosis of brucellosis in patient serum samples. A portable detection system like any hand held potentiostat can be used in field related sensing applications.

IV. CONCLUSION

MWCNTPE is very easy to made and renewable in nature unlike other solid electrodes. In the form of paste, MWCNTs have a number of voids and crevices, which were helpful in trapping the antibodies during the process of physical adsorption as a result of which sensitivity increased several times. In this present article the amperometric response of immunosensor was found

to be linear in the range of 2.5µg/mL to 100 µg/mL and the detection limit of CE-protein antigen was found 1 ng/mL. The relative standard deviation was 7.1%. Whereas by ELISA method, the detection limit of CE-protein antigen was 50 µg/mL. Experiments were also conducted using 110 suspected patient serum samples and results were compared with other standard tests like ELISA & RBPT (Table 1). The immunosensor was found to be more sensitive than standard tests. Post-zone like effect was found in detection of brucellosis. However, no false negative results were obtained by immunosensor and these results were concluded by repeating the experiments on diluted samples. Thus the immunosensor can be used for diagnosis of brucellosis in patient serum samples. A portable detection system like any hand held potentiostat can be used in field related sensing applications.

V. CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Captions of Fig./Graphical abstract/Table

Fig. 1(a). Components of teflon electrode.
Fig. 1(b). Teflon electrode filled with MWCNT-paste.
Fig. 2. CV of MWCNT paste electrode in 1mM K_3FeCN_6 (0.1M KCl) in PBS. Scan rate (50mV/s).
Fig. 3. Relative RAb adsorption efficiency of MWCNT paste electrode and SPE.
Fig. 4. Raman spectra of acid treated MWCNTs.
Fig. 5. Nyquist plot of EIS in a solution containing 1mM $Fe(CN)_6^{3-/4-}$ with 0.1M KCl at 0.20V (frequency range 0.01Hz–10 kHz). (a) represents the spectra of unmodified MWCNT paste electrode and (b) represents spectra of MWCNT paste electrode after immobilization of antibody.
Fig. 6. Optimization of ALP enzyme tagged antibody (Revealing antibody) dilution.
Fig. 7. Immunosensor response of CE-antigen at high concentration and low concentration.
Graphical abstract. Sandwich immunoassay procedure for immunosensing of CE-Antigen using MWCNT-paste electrode.
Table 1. Comparing results of overall patient serum sample analysis done by amperometric immunosensor method and ELISA method (without serum sample dilution and with serum sample dilution).

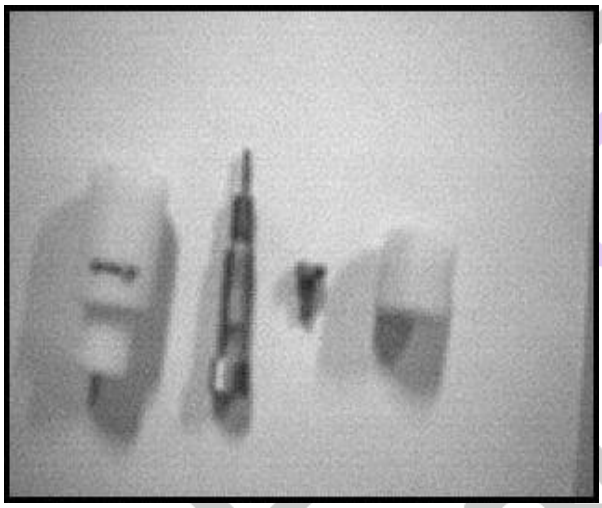


Fig. 1(a). Components of teflon electrode

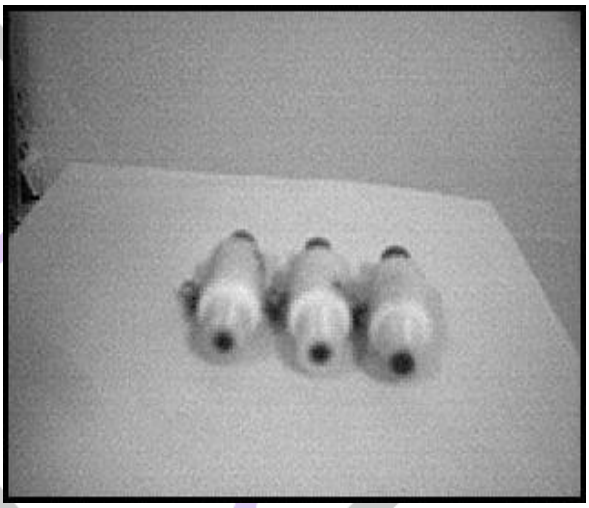


Fig. 1(b). Teflon electrode filled with MWCNT-paste

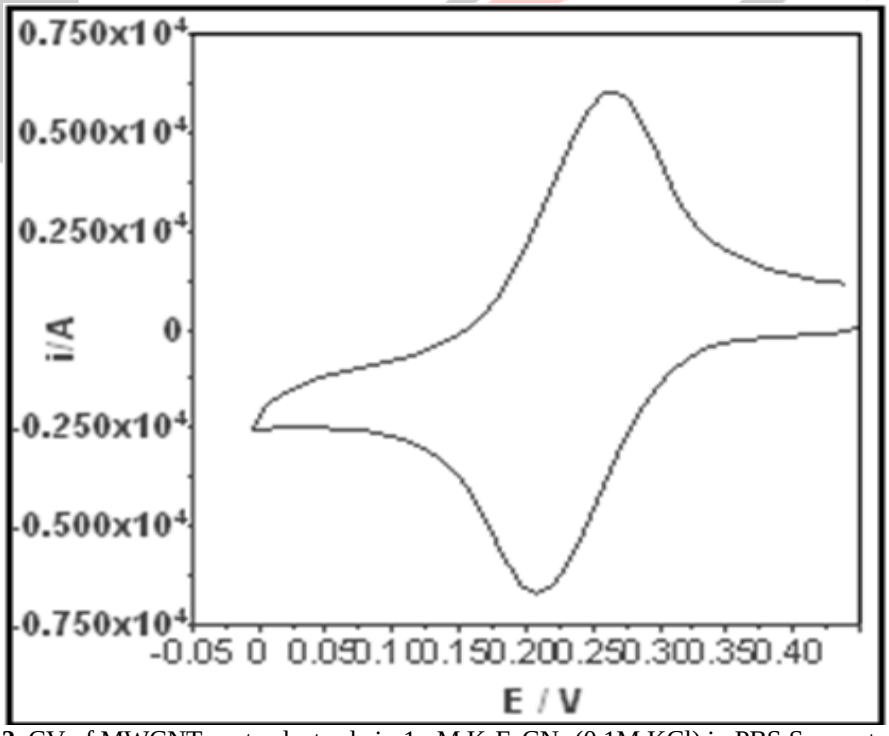


Fig. 2. CV of MWCNT paste electrode in 1mM K_3FeCN_6 (0.1M KCl) in PBS.Scan rate (50mV/s)

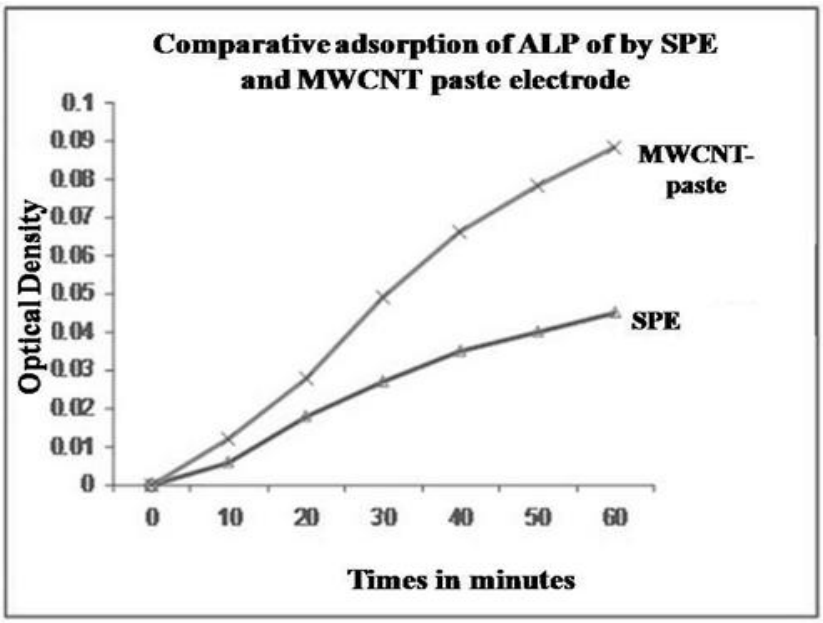


Fig. 3. Relative RAb adsorption effiency of MWCNT-paste and screen printed electrode

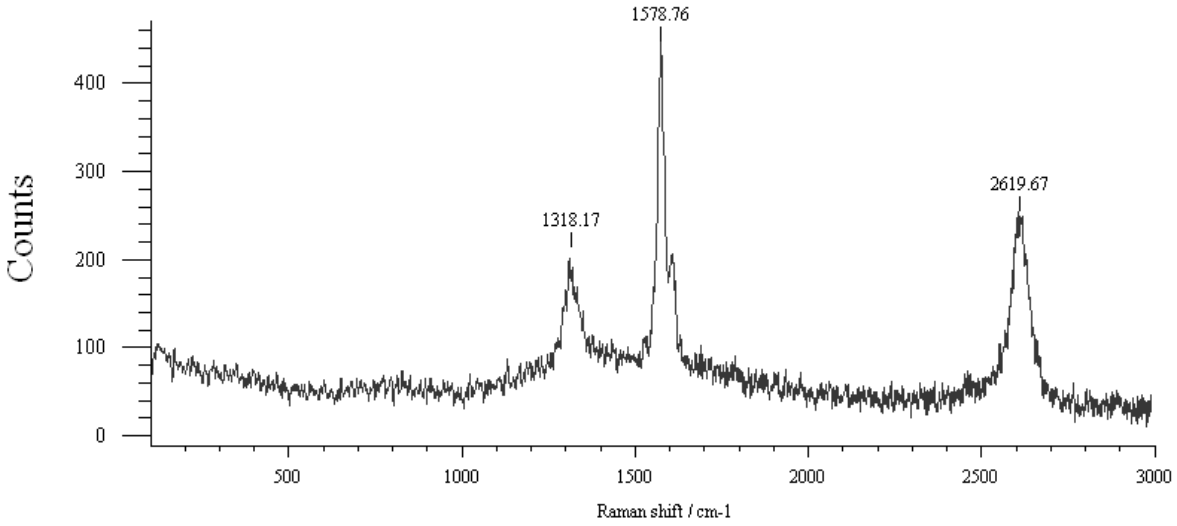
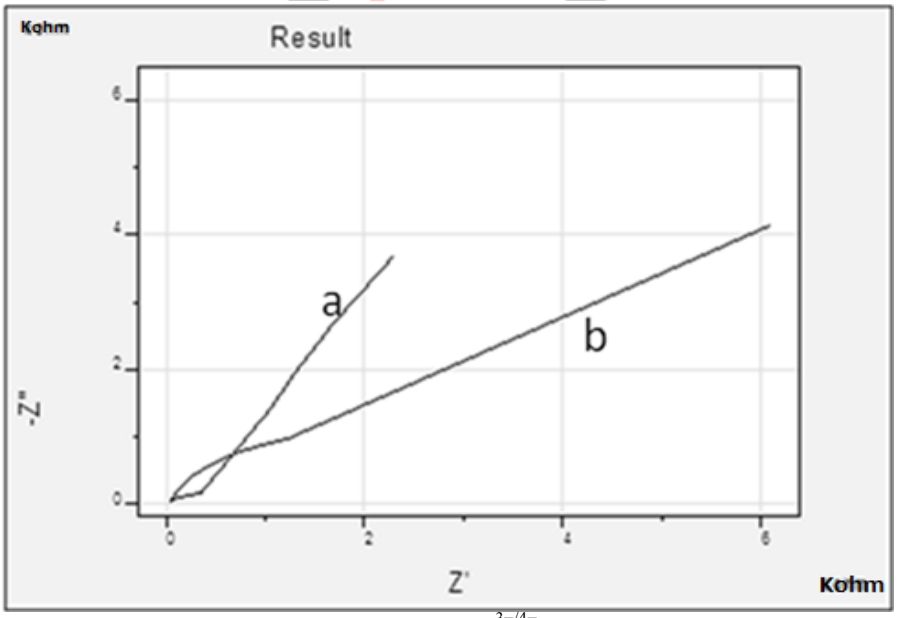


Fig. 4. Raman spectra of acid treated MWCNTs



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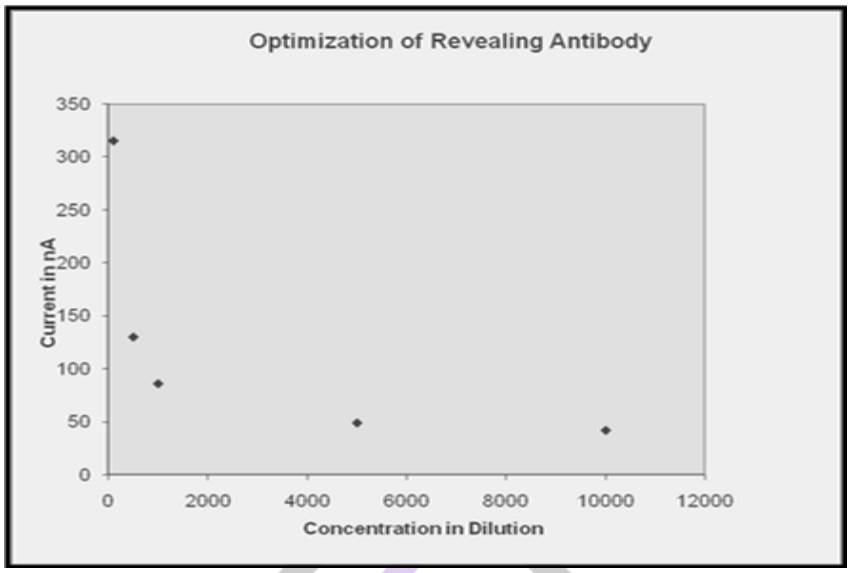

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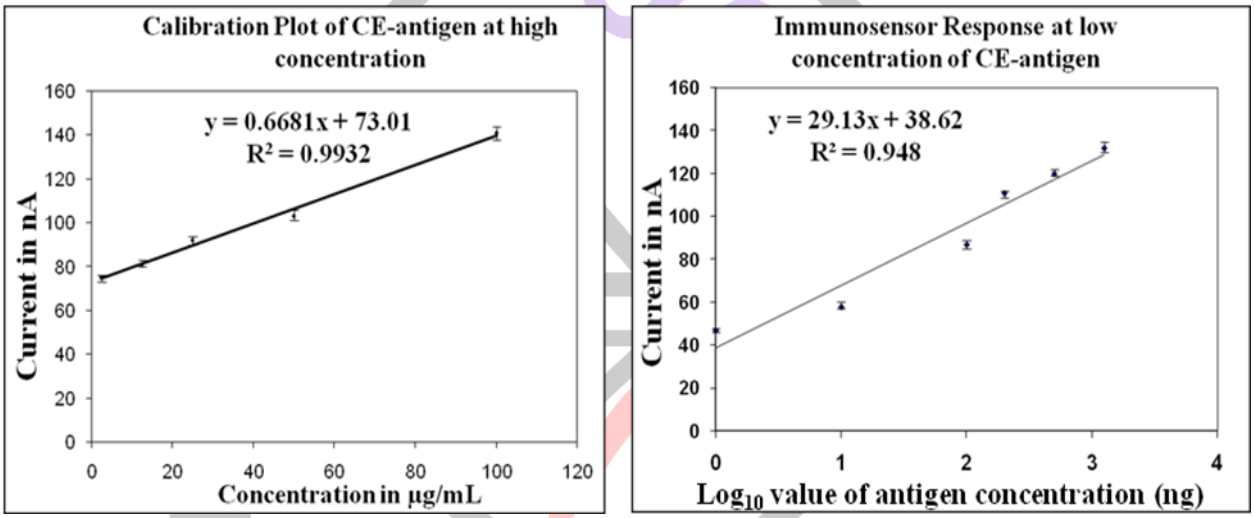
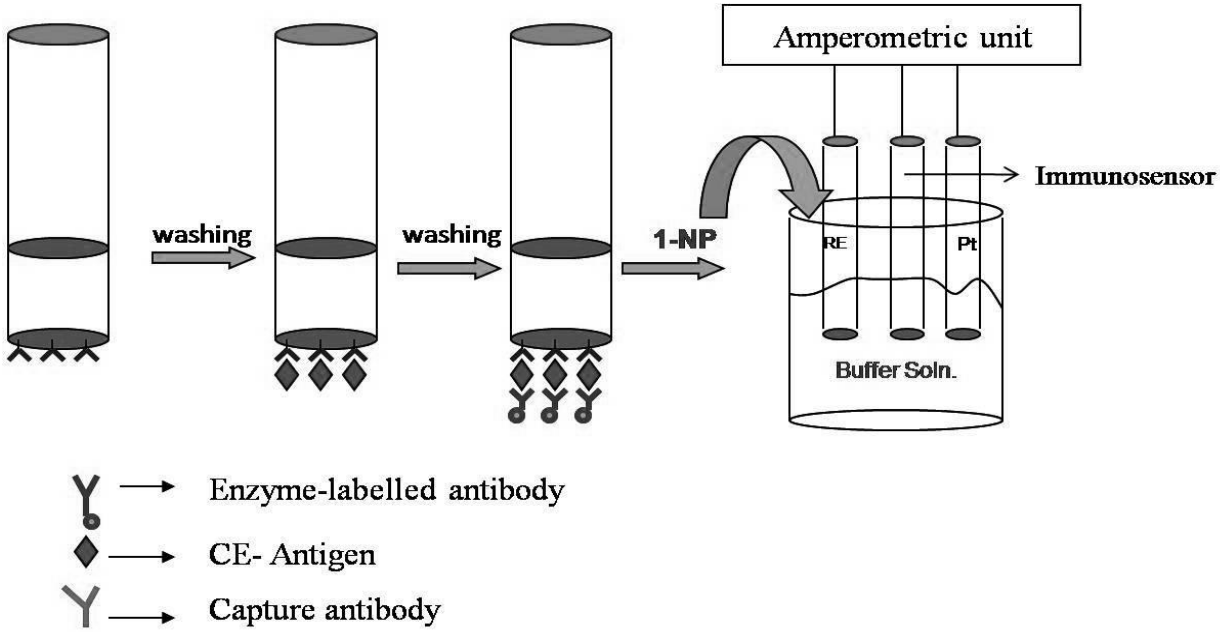


(a) (b)

Fig. 7. Immunosensor response of CE-Antigen at high concentration and low concentration



Graphical abstract. Sandwich immunoassay procedure for immunosensing of CE-Antigen using MWCNT-paste electrode.

Table 1:

Comparing results of overall patient serum samples analysis done by amperometric immunosensor method and other methods like ELISA and RBPT (without serum sample dilution and with serum sample dilution).

	Culture	Without Sample Dilution			
		ELISA Method		RBPT Test	Electrochemical Methods (Antigen Detection)
		Antigen Detection	Antibody Detection		
No. of positive Samples	19	28	39	48	73
No. of Negative Samples	91	82	71	62	33
No. of unclear Samples	-	-	-	-	04
Total No. of Samples	110	110	110	110	110
			Samples after 10 times dilution		
No. of Positive Samples	-	00	12	00	73
No. of Negative Samples	-	110	98	110	37