IJPSR (2015), Vol. 6, Issue 9 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 17 February, 2015; received in revised form, 13 April, 2015; accepted, 05 June, 2015; published 01 September, 2015

IMMOBILIZED BACTERIOPHAGE USED FOR SPECIFIC DETECTION OF *E.COLI* USING ELECTROCHEMICAL IMPEDANCE SENSING

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Keywords:

Bacteriophage, Biosensors, Electrochemical, TEM and Impedance Analysis

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ABSTRACT: In the present study, bacteriophage based electrochemical analysis is applied to detect pathogenic *E.coli* bacteria. Phages are parasites that only infect one specific bacteria, can be useful to identify bacterial contaminants in food, water, etc. Phage has been isolated from various water sources such as waste, stagnant and sewage water by overlay method against host bacteria. Titer of phages was calculated to be 10^7 pfu/ml using 10-fold dilutions. Morphology and identification of phage was done using Transmission Electron Microscopy (TEM) by staining with Uranyl Acetate. The phage capsid was about 78 nanometers (nm) in diameter with tail of length 527 nm as compared to wild type lambda phage whose head is about 65 nm. Plaque reaction activity was observed within 4- 6 hrs against host bacteria by spot test. It is therefore proposed that the isolated phage is lambda like virus and lytic life cycle. Bacteriophages are immobilized onto platinum (pt) electrode by self-assembled molecular monolayer (SAM) and electrochemical impedance analysis performed for a rapid and specific detection of *E.coli* cell. Impedance spectra for sensors of different concentrations of E .coli are recorded in phosphate buffered saline (PBS) and impedance were obtained in a frequency range from 100mHz to 100kHz, using a modulation voltage 10mV. Rapid and Specific detection with very low concentration of *E.coli* (10^4 cfu/ml). This work proposes that measurements of impedance of cell suspensions can provide an alternative method quantifying bacterial cells that is very simple, rapid, economical and a direct means of detecting a specific bacteria.

INTRODUCTION: Billions of people are infected by food and water borne diseases each year worldwide. The detection of such pathogenic microorganisms has gradually become a prime area of interest in field of food and water safety, public health and security [2] of these pathogenic microorganism, bacterial pathogens make up the major cause of death throughout the world, for example: like *Campylobacter jejuni*, *Clostridium botulinum*, *Salmonella* (over 1600 types), *Escherichia coli*, *Listeria monocytogens* and *Shigella* (over 30 types). *E.coli* is one of the major food borne bacteria of utmost concern today, since it can cause severe and sometime life threatening illnesses [24-26].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(9).3913-19

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(9).3913-19

To provide a timely and effective treatment to a susceptible population methods involving rapid, specific and sensitive detection of Pathogen is therefore essential [14, 37]. Conventional methods for detection of pathogens include colony counting, various biochemical methods, immunological assay based methods, PCR methods involving DNA Analysis etc. These may take up to several hours or even a few days to yield an answer. Obviously this is inadequate, and recently many researchers are focusing towards the progress of rapid methods [14]. Thus, there is an immediate need in development of bacterial sensing platforms that ensure timely pathogen detection at low concentration, that are reliable and affordable to use.

Bacteriophages are viruses that have been used as a promising recognition elements to use in bacterial biosensors [3, 30, 33, 34]. They are highly specific to bacteria and harmless to human, less expensive than antibodies and have a longer shelf life. They

can be immobilized onto transducing devices. Electrochemical biosensors are mainly fascinating because they are usually inexpensive and are well adapted to miniaturization[6]. Electrochemical Impedance Spectroscopy (EIS) is a powerful electrochemical technology that is sensitive to small changes occurring at solution-electrode interface [12, 35]. EIS has been widely applied in field of microbiology as a mean to sense and / or quantify pathogenic bacteria [13, 19, 22, 27-29].

EIS being a compact system i.e simple, Rapid and cost effective is an alternate for detection. We apply EIS for Phage based electrochemical detection by use of Self Assembled Monolayer (SAM) and covalent binding, making the process highly specific and sensitive for bacteria. The sinusoidal current obtained in response to small amplitude, sinusoidal perturbations of the potential were measured for the detection over the electrode. Development of impedance biosensors by integrating impedance with biological recognition elements has reduced the detection time down to ~ 30 min [13, 15, 17, 19, 22, 27-29]. EIS using phages has been successful in generating dual signals that involves increase in impedance on bacterial capture and a successive decrease in the same on bacterial lysis [12, 23, 33, 34].

In this paper investigation were carried out to isolate and characterize the *E. coli* specific bacteriophage from waste water sample. Isolated bacteriophage was immobilized on pt-electrode by SAM method, this strategy allowed *E. coli* detection with a limit of detection (CFU/ml) by electrochemical impedance analysis.

MATERIAL AND METHODS:

Bacteria:

E.coli MTCC-1585 were grown in Luria-Bertani (LB) medium (Hi-Media, India) for 16-20 h at 37^{o}C. Cells were tittered using culture plate colony count and found approximately 2 x 10^4 cfu/ml. 1ml of the stock was subjected to serial dilutions in sterile phosphate buffered saline (Hi-Media, India) (120mM NaCl, 50mM NaH_2Po_4, pH 7.4) [18].

Isolation of phage, enrichment and overlay method: Equal amount of samples were centrifuged at 3000 to 5000 rpm for 15 min at room temperature to remove any large debris and insoluble waste. The supernatant was filtered through a sterile strainer (0.45 µm filter, Millipore, India) [18]. Filtrate sample containing phages was added to log-phase *E.coli* culture broth for 5 to 24 h for incubation in a shaking incubator having shaking speed of 200 rpm at 37^{o}C for phage enrichment.

Solution was again centrifuged at 5000 rpm for 15min at room temperature and the supernatant re-filtered to remove any cell debris to obtain final enriched phage suspension (0.45 µm filter, Millipore, India) [8, 18]. The presence of any phages in the enriched filtrate was carried out by overlay method (Adams, 1959)[1] with some modifications. Briefly, 1 ml filtrate was added to tubes containing 5 ml of soft agar (0.7 g Agar Hi-media, India) in 100ml of LB broth mixed well poured onto LB Agar plates were then solidified and incubated overnight in incubator at 37^{o} C. The incubated plates were observed for the presence of plaques at every 1 h interval.

Plaque picking, spot test and plaque forming unit:

The visible plaques were picked from each soft agar Plates (well isolate zone) using sterile pipette tips in Phosphate buffer saline (PBS) (Hi-media, India) after that PBS solution was filtered using 0.45 µm filter (Millipore, India) to remove bacteria, media and cell debris and was stored at 4°c. The presence of phages was confirmed with the help of spot test, this has also been reported in literature [8] .

A mixture of top agar and overnight grown culture of *E. coli* was spread over it. The 10- fold serially diluted solutions of filter sterilized phages were prepared in PBS 2-4 µl of each of the dilution of phage suspension was spotted over the plate. The plate was incubated at 37˚ C and observed for clear zone of lysis. Plaque forming unit (PFU/ml) of phage was calculated [8].

Electron microscope studies on phage:

100ml of phage stock was revived for ultracentrifugation (Backmen Optimal LE-80k UC) T90i rotor fixed angle, Ultracentrifuge system. Phage sample were loaded in 8 number tube which is specially designed for this system & each

contained 10 ml at 45000rpm for 2h at 4°C. Supernatant was collected in different tubes and pellet was dissolved in SM buffer (5.8g NaCl, 2g $MgSO_4.7H_2O$, 50ml/l of 1M Tris pH 7.5, 5 mL/L of 2% gelatin in distilled Water) and stored at 4° C [18].

SM buffer containing phage was negatively stained and Preparation was used to examine the structure by electron Microscopy. 100µl of sample was applied to the surface of a 200 mesh copper grid, negatively stained with 2.5% Uranyl acetate, and then examined in a FEI TECNAI (G2)F-20 transmission electron microscope operated at 120KV.The stained grids were viewed and photographed under the electron microscope. These images displayed phage tail length and capsid Diameter, both of which were helpful in determining the phage's cluster. Images were taken at x29000 resolution.

Modification of electrode:

Immobilization of bacteriophage was based on the formation of self-assembled monolayer on Platinum (Pt) electrode surface (2mm diameter AUTOLAB, Metrohm). A schematic diagram of the process is illustrated in **Fig.1**. Briefly, the Pt electrode was immersed in a 1mM ethanolic solution of 11-mercaptoundecanoic acid (MUA) (Sigma) for 16-20 h at room temperature. Further, the electrode surface was washed with deionized water (DI) to remove any unattached species and then dried in a stream of nitrogen. The pt electrode was activated in an aqueous solution of (0.1g/ml ddH_2O) 1-(3-dimethylaminopropyl) – 3 - ethyl-carbodiimide (EDC) (Hi-media, India) and 0.1 M n-hydroxysuccinimide in ddH_2O(NHS) (Sigma) for 1-2 h at Room Temperature; washed with deionized water (DI) and dried with nitrogen. Pt Electrode was dipped in 1 ml of isolated bacteriophage & then incubated at room temperature for 1-2h.

Electrode surface was then washed 3 times with PBS buffer (Hi media) (pH 7.4), Followed by 10µl of Bovine Serum Albumin (BSA) (Sigma) solution (1%) and incubated for 20 minutes to prevent non-specific adsorption of non-targeted bio-components [7, 10, 21, 22].

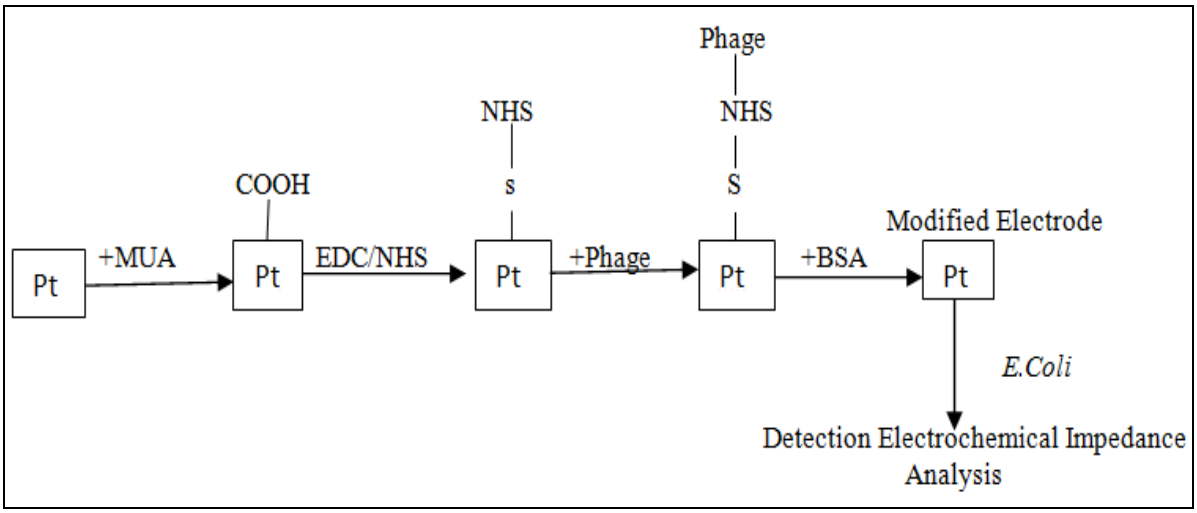

FIG.1: SCHEMATIC ILLUSTRATION OF THE PREPARATION OF THE PHAGE BIOSENSORS

Assay performance:

E. coli overnight culture was 10 fold serially diluted in PBS (10^0 to 10^4 CFU/ml) to determine the limit of detection of *E.coli*, that was spotted over the modified Pt disc electrode [7].

Electrochemical Apparatus:

Electrochemical measurements were performed at room temperature in a voltammetric cell with three-electrodes, electrochemical cell configuration with a pt disc working electrode, a platinum wire as the counter electrode, and an Ag/AgCl (filled with 3M KCl) reference electrode. We used an (AUTOLAB PGSTAT 302N, Netherland) cyclic voltammetry and FRA impedance analyser equipped with the NOVA 1.10 acquisition software. Cyclic voltammetry (CV) was carried out using same electrodes with Potential scanning from -0.2 to +0.6 V at a scan rate of 100mV/s in order to determine the midpoint between the oxidation and

the reduction of the redox couple, which can be used as an applied DC potential for further impedance measurements. The impedance were obtained in a frequency range from 100mHz to 100kHz, using a modulation voltage 10mV complex plane diagram (Nyquist plot) with a sampling rate of 5points per decade. The CV and impedance measurements were performed in PBS buffer (pH 7.4) in the presence of 10 mM of **Of** $Fe(CN)_6^{3-/4-}$. [4, 5, 7, 16, 20].

RESULT AND DISSCUSION:

Isolation of Bacteriophage:

Bacteriophages specific for *E. coli* were isolated from Waste water and stagnant water sample from Haryana as evident by clear zones **Fig.2**. Plaques appeared as clear zones found in confluent bacterial growth. Turbidity signified the presence of lytic phage. The number of phage in a solution were determined by calculating the number of plaque forming units per milliliter of phage. The plaques were clearly identified on *E. coli* plates **Fig.2**. Plaque forming unit (PFU) was calculated by number of infected virus particles per unit volume 10^7 PFU/ml.

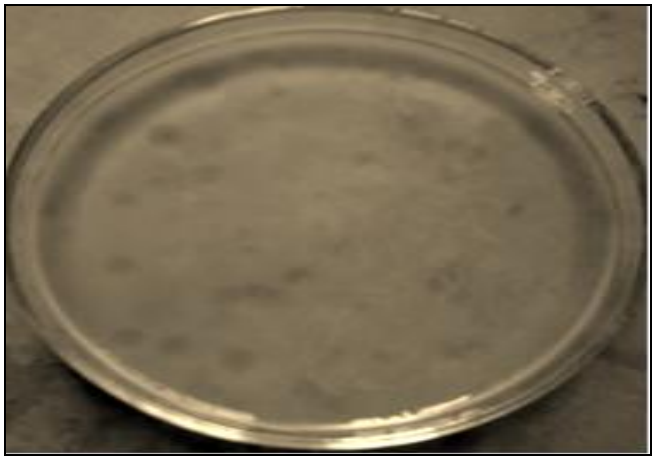

FIG.2: PLAQUE FORMATION IN *E. COLI* WHEN INFECTED BY ISOLATED PHAGE

Phage morphology and taxonomic classification:

Characterization and identification of family of phage was done using Transmission electron microscopy (TEM) by staining with Uranyl Acetate **Fig.3**. The phage head or capsid was about 78 nanometers (nm) in diameter with long tail of length 527 nm. Isolated phage was classified into order *Caudovirales* and as it possesses a long noncontractile tail and icosahedral capsid head, thus is a member of the family *Siphoviridae*. In present investigation it was observed that the isolated phage seemed similar to lambda like virus phage [36].

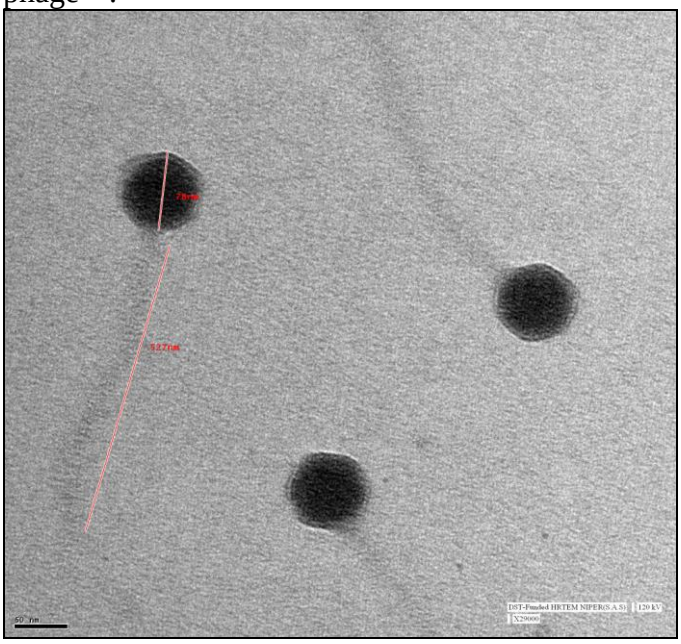

FIG.3: ELECTRON MICROGRAPHS OF ISOLATE BACTERIOPHAGE STAINING WITH URANLY ACETATE (AT X29000)

Cyclic voltametery:

The Cyclic voltametery is an analytical method that is used for rapid surface modification and initial characterization. The reversible redox couple, 1mM $Fe(CN)_6^{3-/4-}$, in Deionized water, was selected as a redox probe to study the characteristics of the pt electrode.

Fig.4(a) shows the cyclic voltammograms of the pt electrode in $Fe(CN)_6^{3-/4-}$ solution after different modification steps. $Fe(CN)_6^{3-/4-}$ showed a reversible behavior on pt electrode with peak-to-peak separation of 100mV/s. The self-assembly of the MUA and activation of COOH end groups are supplemented by a decrease in the peak current and resistance increases due to modification in covering of the electrode at different dilution (10^1 to 10^4 cfu/ml) **Fig 4(b),** determined by the cyclic voltammetry curve of pt working electrode, this has also been reported in literature [10, 21].

Cyclic voltammetry of modified electrode surface scanned at potential from -0.2 to +0.6 V at a scan rate of 100mV/s. The formal potential of 0.20 V was estimated from the mean of the anodic and cathodic peaks of the cyclic voltammogram of the

bare pt electrode **Fig 5**, the decreases in the peak currents might be attributed to the fact that the bacteriophage (black) and MUA/EDC/NHS (blue) insulated the surface and effectively enhanced the electron transfer barriers. The reason might be due to the barrier of the assembled layer that stops the access of redox Couple also Couple and also low current involved in the redox reaction after functionalization of the pt surface with MUA[7].

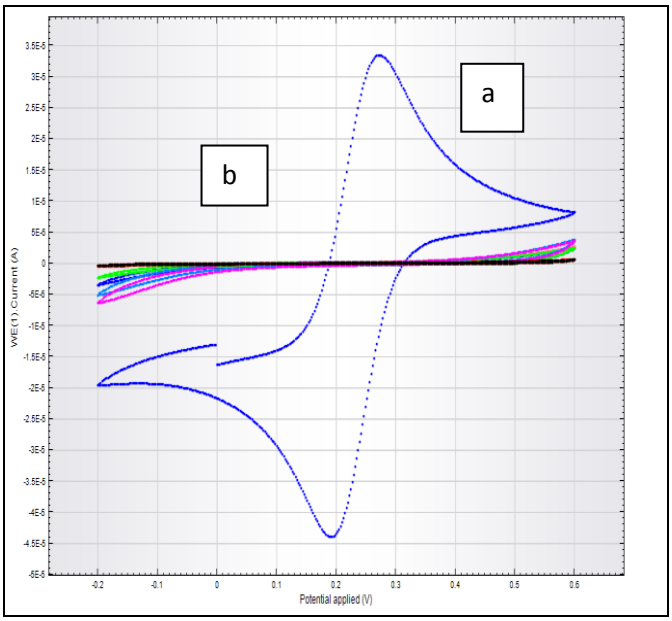

FIG.4: CYCLIC VOLTAMETRY RECORDED IN PBS, 1MM POTASSIUM FERRIC CYANITE, AS REDOX ACTIVE AT SCAN RATE OF 100MV/S A) PT ELECTRODE AND B) PT ELECTRODE MODIFIED WITH A MUA, DIFFERENT BACTERIAL CONCENTRATION OF *E.COLI*. (10^1 CFU/ML TO 10^6 CFU/ML)

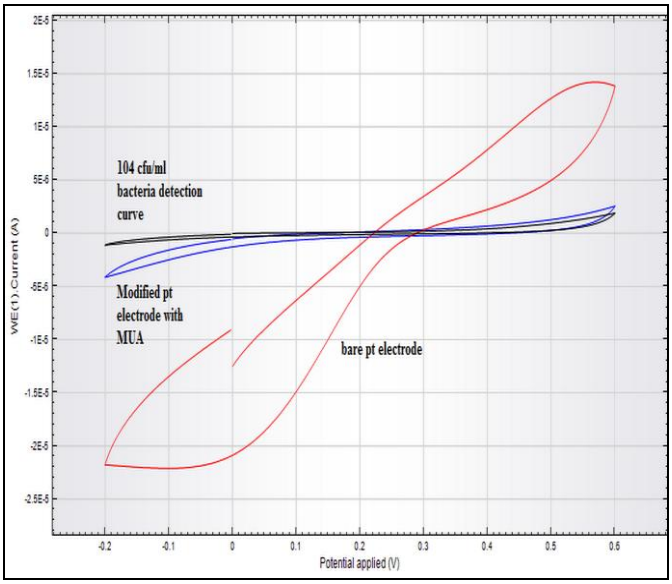

FIG.5: CYCLIC VOLTAMMETRY OF BARE PT ELECTRODE (RED LINE), MODIFIED PT ELECTRODE WITH MUA REDOX ACTIVE $FE(CN)_6^{3-/4-}$, (BLUE LINE) PBS SOLUTION, (BLACK LINE) OF 10^4 CFU/ML CURVE.

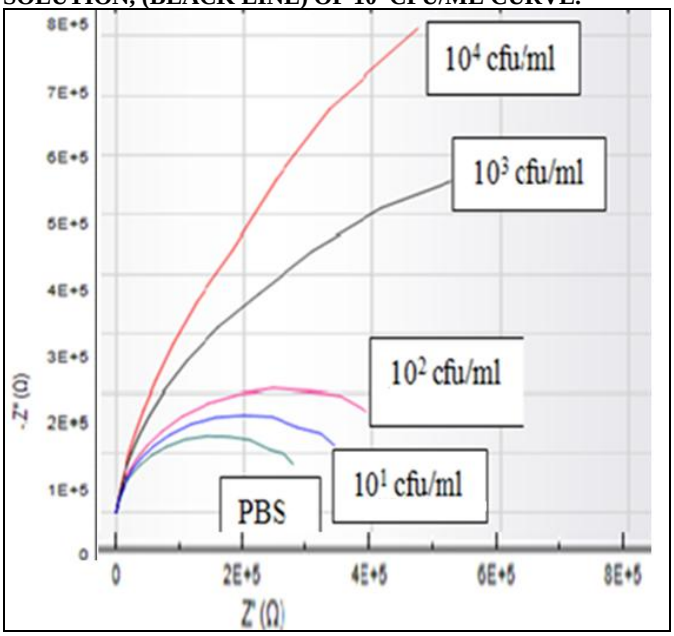

FIG.6: NYQUIST PLOTS OF IMPEDANCE SPECTRAS OBTAINED FOR INCREASING CONCENTRATIONS OF E.COLI FROM 10^0TO 10^4 CFU/ML IN PHOSPHATE BUFFERED SALINE (PBS) PT ELECTRODE WITH PHAGE IMMOBILIZED BY COVALENT BONDING.

Electrochemical impedance studies:

The impedance spectra showed a semicircle and linear portion. The semicircle portion at higher frequencies corresponds to the electron-transfer limited process, and the linear portion at lower frequencies represents the diffusion-limited process [21]. Phage modified Microelectrode is kept in the presence of *E.coli* (10^4 CFU/ml) in PBS solution and a frequency range between 100mHz to 100kHz [31, 32]. Impedance Analysis is carried out for the detection of *E.coli* bacteria in apparatus cell, containing sterile PBS with different bacterial concentration (10^0 to 10^4 cfu/ml).

All tests were performed at room temperature. Fig.6 Nyquist plot curve shows an initial Increase in the impedance (8×10^5) that proceeds for about 4min, impedance increases slowly over time until it stabilises. The semicircle corresponds to the charge transfer resistance of the electrode interface [12]. Observed decrease of the charge transfer resistance is due to the increase in conductivity after bacteriophage immoblization.

This decrease of the charge transfer resistance is due to the specific bacteria and bacteriophage recognition. The subsequent decrease in impedance

presumably happens as a consequence of phage-induced bacterial infection and lysis, which induces cell wall disruption and release of important amounts of intracellular Components, this has also been reported in literature [19]. This event induces increase of the medium conductivity at the vicinity of the electrode surface, which contributes to decrease charge-transfer Resistance, this has also been reported in literature [9, 11].

CONCLUSION: In the present study, we isolated bacteriophage from different waste water sources against *E.coli* and thereafter characterized it by TEM. It was classified as lambda like virus. Biosensor development strongly depends on the optimisation of surface functionalisation strategies using bacteriophage based bio component for bacterial bio sensing. Self-assembly monolayer Suggests that surface functionalisation for the specific detection of whole microorganism. The produced surface has been applied to bacterial detection by impedance analysis.

The change in the electron transfer resistance was due to phage immobilization and binding of *E.coli*. The specificity of the immunosensor was tested against *E.coli* bacteria. A limit detection of 10^4 CFU/mL was obtained with a good reproducibility. For future work, this is a generic platform for advanced phage mediated bacterial sensing system, with a high promise in practical applications.

ACKNOWLEDGMENT: The authors would like to thank Director UIET, University Institute Engineering and Technology, Kurukshetra University, Kurukshetra, Kurukshetra University, Haryana, India for providing the infrastructure and other facilities to carry out this project. The authors are also grateful to NIPER, Mohali, India for helping out in using their facilities. Scholarship-fellowship by RGNF-UGC, India.

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How to cite this article:
Singh V, Rawal V, Lakhanpal S, Jain P, Dahiya S and Tripathi CC: Immobilized Bacteriophage Used For Specific Detection of *E.Coli* Using Electrochemical Impedance Sensing. Int J Pharm Sci Res 2015; 6(9): 3913-19.doi: 10.13040/IJPSR.0975-8232.6(9).3913-19.