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Full Length Research Paper

Bacteriophage based self-assembled monolayer (SAM) on gold surface used for detection of *Escherichia coli* by electrochemical analysis

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Bacteriophages (or phages) are parasites that infect specific bacteria. This host-selectivity can be useful to identify bacterial contaminants in food, water, environment etc. In the present study, phage was isolated from stagnant water and cultivated by overlay method against host bacteria that is *E. coli*. Phage titer was calculated to be 10^7 pfu/ml using 10-fold dilutions. Plaque reaction activity was observed within 4 to 6 h against host bacteria by spot test. Morphological identification of phage by transmission electron microscopy (TEM) using uranyl acetate staining revealed about 78 nanometers (nm) in wide phage capsid and tail length (527 nm). The isolated phage was classified into order *Caudovirales* since it possessed a long non-contractile tail and icosahedral capsid head, thus is a member of the family *Siphoviridae*. Also, the phage identified followed a lytic life cycle since plaque reaction activity was observed within 4-6 h against host bacteria. Gold immuno-functionalization using self-assembled monolayers (SAM) has been widely used for the detection of small targets, but there are limited reports available describing the detection systems for bacteria by using phages. Thus, in order to develop a suitable detection system for identification of specific bacteria, it is suitable to exploit the close association and selectivity between bacteria and bacteriophage. Bacteriophages were immobilized onto gold surface by SAM using a stable acyl amino ester intermediate generated by 1-ethyl-3(3-dimethylaminoproply) carbodiimide (EDC) and N-hydrosuccinimide (NHS) to condense the bacteriophage. Fourier transformation infrared (FTIR) microscopy presence of different functional groups present in each layer formation. Bacteriophage immobilization over the gold surface was verified through by scanning electron microscope (SEM). Electrochemical analysis was performed for a rapid and specific detection of *E. coli* cell. The present bio-sensing system comprises of quick and specific detection of host bacteria and possesses a very low detection limit (10^4 cfu/ml). We propose phages utilization as a bio-component in biosensor development for bacteria capture.

Key words: Bacteriophage, biosensors, electrochemical, microscopy, immune-functionalisation and Self-assembled molecular monolayer.

INTRODUCTION

Bacteriophages are highly specific viruses that infect bacteria and are essentially harmless to humans. High host-specificity and selectivity promotes distribution of virions in locations populated by bacteria, such as natural water bodies, soil and intestinal tracts of warm-blooded animals.

Escherichia coli is a natural resident in the intestinal tracts of animals including humans. *E. coli* can cause diarrhea, food poisoning, inflammations etc. also major cause of foodborne illness (Geng et al., 2008).

Bacterial infection has been one of the major causes of outbreak of diseases and hence a major threat for human health and food safety for decades (Velusamy et al., 2009). Conventional methods for detection of these pathogens include colony counting, biochemical methods, immunological assays, PCR methods involving DNA analysis etc. These conventional methods may take from several hours to even a few days to yield an answer. Thus, there is an immediate need for developing sensitive, selective, reliable and quick bacterial sensing platforms which ensure pathogen detection at low concentration, and are affordable to use (Chemburu et al., 2005). In this regard, researchers are now-a-days focusing towards development of rapid detection methods (Petty et al., 2006; Alocilja and Radke, 2003; Naidoo et al., 2012).

Bacterial biosensing platform is based upon the use of detecting the presence of bacteriophage cross-linked to gold surface itself, instead of selective antibody identification. During infection, the bacteriophage binds to specific receptors on the host bacterial surface. Following injection of the phage genome into the bacteria, high copy-number replication of phage takes place in the host bacterial cell, and subsequently, several new phages are released. Phage production is possible in an easier, faster, and cheaper, which makes them promising tools in biosensing (Johnson and Zeikus, 1991; Balasubramanian et al., 2007; Zeikus and Johnson, 1991; Yang and Bashir, 2008). They can also be immobilized onto transducing devices which makes detection more rapid biosensor platforms being developed for electrochemical analysis by impedometric-based detection (Zourob, 2010; Rohrbach et al., 2012).

Electrochemical impedance spectroscopy (EIS) is of great attention since it is proficient and frequent in detecting small change occurring at the solution-electrode interface and also corresponds to the charge transfer resistance of the electrode/electrolyte interface. Furthermore, EIS is simple, cost effective and processing is less reagent-dependent (Mejri et al., 2011). Recently, use of EIS has been broadly applied in the field of microbiology as a means to sense and measure pathogenic bacteria (Mejri et al., 2011; Mejri et al., 2010; Rohrbach et al., 2012; Park et al., 2013).

Modification or functionalization means the transformation of a biologically-inert material into a bioactive or bio-functional material. When gold surface are considered, preferred strategies via self-assembled monolayer (SAM) and covalent binding provide options for strong and stable biomolecules immobilization in the very near vicinity of the sensor surface (Yang and Bashir, 2008; Shabani et al., 2008; Mejri et al., 2010, Yang and Li, 2005; Yang et al., 2004; Ruan et al., 2002; Radke and Alocilja, 2005). As gold surface is relatively homogeneous and provides suitable reactive groups for the directed conjugation of capture biocomponents it is used as a substrate (Baldrich et al., 2008). Various researchers have used this strategy for bacterial detection and there have been many successful reports in which a SAM-based immuno-sensor enables the detection of bacteria (Patel et al., 1997; Baldrich et al., 2008; Geng et al., 2008; Schofield et al., 2012).

In present investigation, experiments were carried out to isolate and characterize the *E. coli* specific bacteriophage from waste-water sample. Isolated bacteriophage was immobilized on modified gold (Au) electrode by SAM method. Mercaptoundecanoic acid was used for self-assemble on Au surface to form an oriented monolayer. The immobilization of bacteriophage on the SAM was carried out through a stable acyl amino ester intermediate generated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydrosuccinimide (NHS) cross-linking reaction. The co-addition of EDC and NHS can provide the formation of an appropriate intermediate to condense bacteriophage on the SAM. Gold surface modification identify was by screening electron microscopy. This strategy allowed *E. coli* detection with a limit of detection of 10^4 CFU/ml by electrochemical impedance analysis.

MATERIALS AND METHODS

Bacteria

E. coli MTCC-1585 were grown in LB medium (HI-Media) for 16-20 h at 37°C. Cells were titered using culture plate colony count and found approximately to be 2 x 10^4 CFU/ml and served as stock culture. Thestock culture (1 ml) was subjected to serial dilutions in sterile phosphate buffered saline (PBS, 120 mM NaCl, 50 mM NaH_2Po_4, pH 7.4) (Sambrook et al., 1989).

Isolation of phage, enrichment and overlay method

Waste water sample were centrifuged out at 3000 to 5000 rpm for 15 min at room temperature to remove any large debris and insoluble waste. The supernatant containing phages was filtered through sterile filters (pore size 0.45 µm, Millipore) and was added to log phase *E. coli* culture broth for 5 to 24 h in a shaking incubator (200 rpm at 37°C) for phage growth (Sambrook et al., 1989). The broth was centrifuged at 5000 rpm for 15 min at room temperature and the supernatant was re-filtered to obtain the final enriched phage suspension (Sambrook et al., 1989; Jordan et al., 2011). The presence of phages in the enriched filtrate was validated by overlay method with some modifications (Adams, 1959). Briefly, 1 ml of

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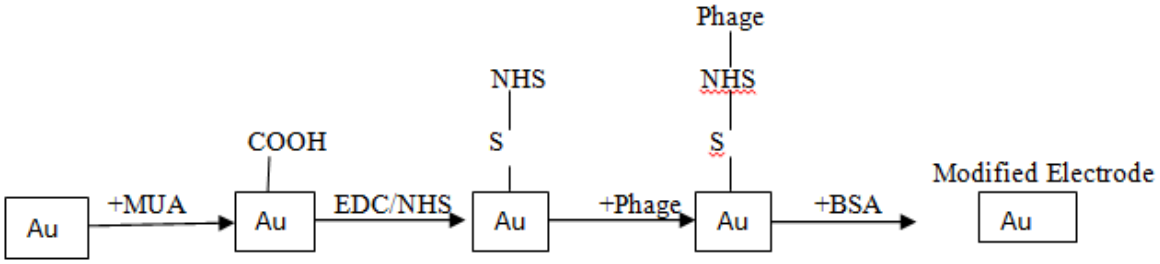

Figure 1. Schematic illustration of the preparation of the phage biosensors.

filtrate was taken and added to tubes containing 5 ml of soft agar (0.7 g/ml, Hi-media) in 100 ml of LB broth). This was mixed and poured onto LB agar plates, solidified and incubated overnight at 37°C. After incubation, presence of plaques was observed at every one hour interval.

Plaque picking, spot test and plaque forming unit

The visible plaques were picked using sterile tips from each soft agar plate (well isolate zone) and transferred to PBS which was then filtered using 0.45 µm filter (Millipore) to remove bacteria, media and cell debris stored at 4°C. Spot test confirmed the presence of phages (Jordan et al., 2011). An overnight grown culture of *E. coli* and top agar was mixed and spread over it. Filter sterilized phages were 10-fold serially diluted using 2-4 µl phsophate buffer saline (PBS) each of the serially dilution of phage suspension being spotted over the plate. Clear zone of lysis were observed after incubation at 37°C and phage density (PFU/ml) of phages was calculated (Jordan et al., 2011).

Electron Microscope

100 ml of phage stock was revived by ultracentrifugation (Backmen Optimal LE-80k UC) at 45000 rpm for 2 h at 4°C. The supernatant was isolated and stored at 4°C and the pellet dissolved in SM buffer (5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 50 ml/l of 1M Tris pH 7.5, 5 mL/L of 2% gelatin in distilled Water) (Sambrook et al., 1989).

Phage in SM buffer was negatively stained and preparations were made to examine the structure by Transmission electron microscopy. On the surface of 200 mesh copper grid (100 µl) of sample was applied and then viewed under microscope negatively operating at 120 kV. The stained grids were viewed and photographs were taken at 19000X, and 29000X magnification, visualizing phage tail and capsid for determining the phage's cluster.

Modification of electrode

Immobilization of bacteriophage was based on the formation of SAM-Au surface (Figure 1). Au was immersed in 1 mM ethanolic solution of 11-mercaptoundecanoic acid (MUA) for 16 to 20 h at room temperature. Washing of electrode was performed using deionized water (DI) to remove any unattached species and the dried under nitrogen stream. Activation of gold surface was carried out in aqueous solution of (0.1 g/ml ddH_2O) 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC) (Hi Media) and (11.5 mg/ml ddH_2O) N-hydroxysuccinimide (NHS) (Sigma) for 1-2 h at room temperature. Again washing was done using deionized water (DI) and dried in steam of nitrogen. Incubation of Gold surface immersed deep in 1 ml of isolated bacteriophage was done at room temperature for 1-2 h. Washing of electrode surface was carried out 3 times with PBS buffer (Hi Media) (pH 7.4), then with 10 µl of Bovine serum albumin (BSA) (Sigma-Aldrich) solution (1%) and incubation done for 20 minto prevent non-specific adsorption of non-targeted bio-components (Lucarelli et al., 2005; Geng et al., 2008; Tolba et al., 2010; Naidoo et al., 2012; Tlili et al., 2013).

Scanning Electron Microscope and FTIR

SEM images was taken at different gold surface condition for bare gold surface, gold substrates was modified with MUA/EDC/NHS and then 20 µL of Phage (10^7pfu/mL) was placed on the modified surface air-dried for 12 min. The images were obtained with the SEM instrument (Hitachi S-3400N, Japan) (Shabani et al., 2008). FTIR spectrum taking using model Perkin Elmer (spectrum 400) under 400 to 4000 wavelength of Gold surface (Rawson et al., 1989).

Electrochemical apparatus

Electrochemical measurements were performed at room temperature in a voltammetry cell with three-electrodes, electrochemical cell configuration with a Au disc (1cm dia) 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 analyzer equipped with the NOVA 1.10 acquisition software. Cyclic voltammetry (CV) was carried out using same electrodes with potential scanned from -1.0 to 1.0 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 was obtained in a frequency range from 100 mHz to 100 kHz, using a modulation voltage 10 mV complex plane diagram (Nyquist plot) with a sampling rate of 5 points per decade. The CV and impedance measurements were performed in PBS buffer (pH 7.4) in the presence of 10 mM of $Fe(CN)_6^{3-/4-}$ Standard (Rawson et al., 1989; Busalmen et al., 2008; Geng et al., 2008; Baldrich et al., 2008; Shabani et al., 2007).

RESULTS AND DISSCUSION

Isolation of bacteriophage

Bacteriophages specific for *E. coli* were isolated from stagnant waste water sample from food industry, Haryana as evident by clear zones of plaque (Figure 2).

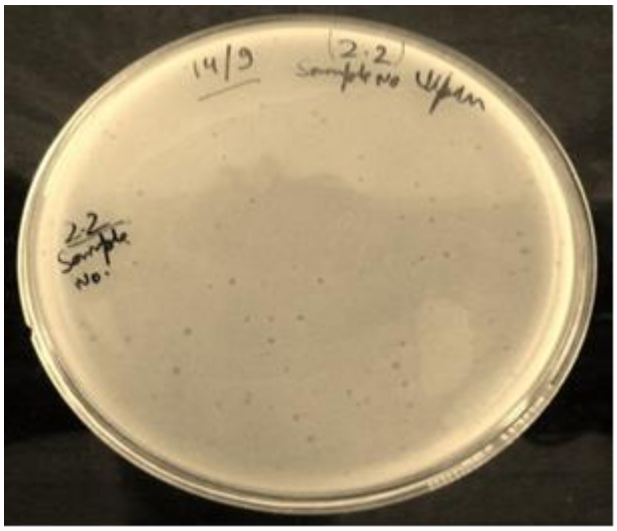

Figure 2. Plaque formation against *E.coli* by agar overlay method.

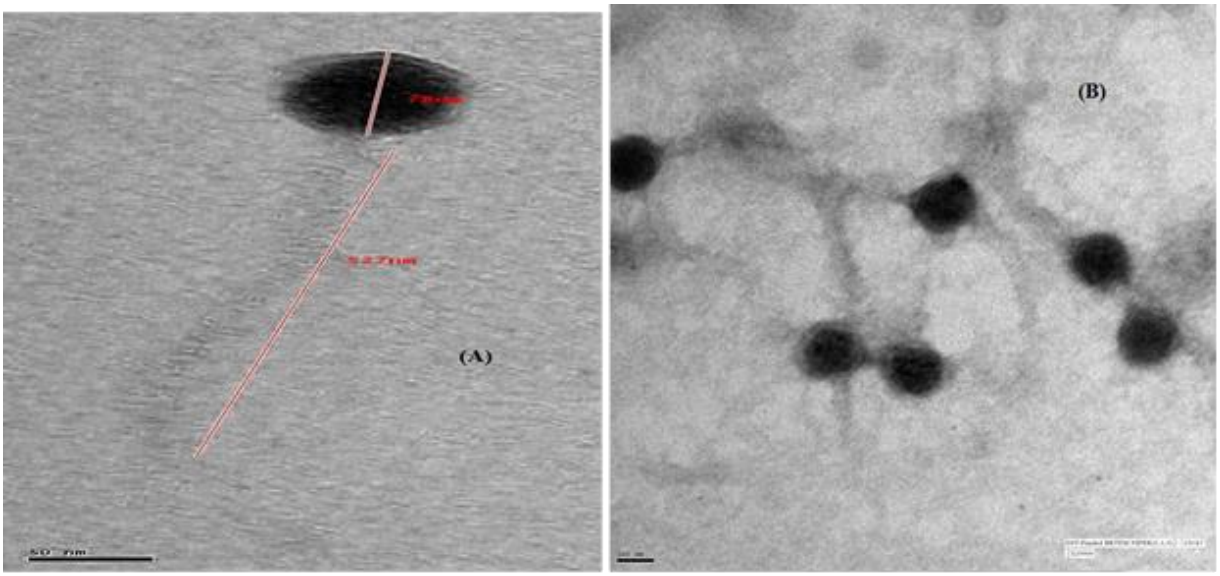

Figure 3. Electron micrographs of isolated bacteriophage stained with uranly acetate (at (A) x29000 and (B) x19 000).

Plaques appeared as clear zones found in confluent bacterial growth by agar overlay method. Turbidity signifies the presence of lytic phage. Plaque forming unit (PFU) was calculated by counting number of infected virus particles per unit volume (10^7 PFU/ml).

Phage morphology and taxonomic classification

Morphology and identification of family of phage was done using TEM by staining with Uranyl Acetate (Figure 3A and B). 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 non-contractile tail and icosahedral capsid head, thus is a member of the family *Siphoviridae*. In the present investigation it was observed that the isolated phage seemed similar to lambda like virus phage, lambda phages specific also called a enterobacteria phage λ that is *E. coli* (Hulo et al., 2011).

Gold surface characterization by SEM

The SEM images shows the surface modification by

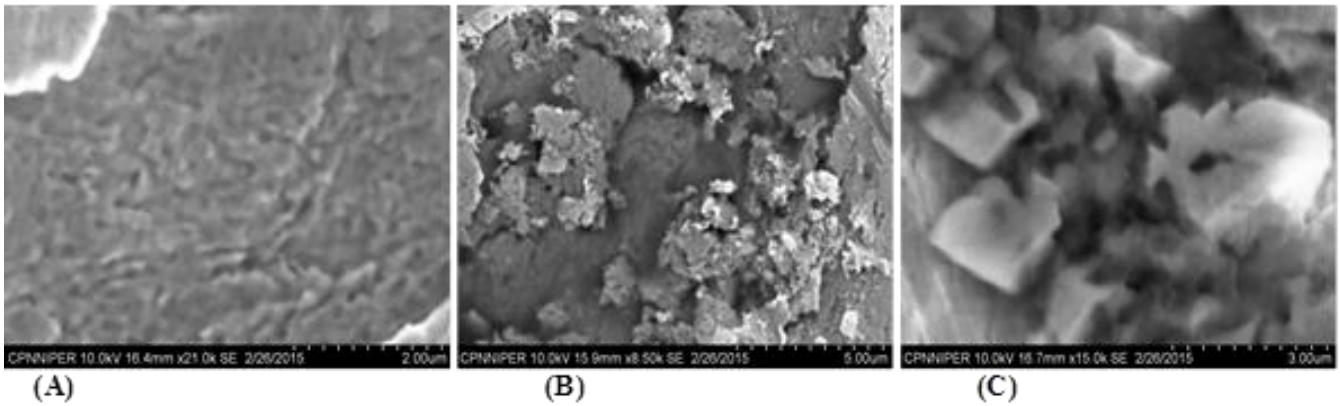

Figure 4. SEM images of phage-modified gold surface. **(A)** Bare gold surface. **(B)** Modified gold surface MUA/EDC/NHS (low resolution). **(C)** Immobilized phage on modified gold surface (low resolution).

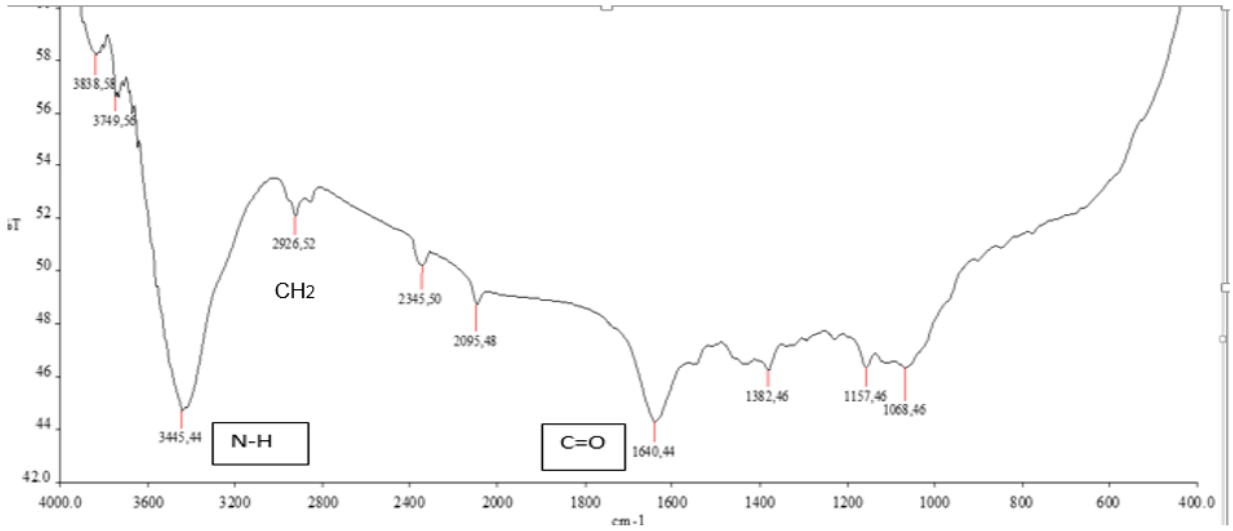

Figure 5. Fourier Transform Infrared Spectrum of the Au/MUA/EDC-NHS electrode assembly. N-H, C=O and CH2 peaks.

self-assembly monolayer on Gold surface (Figure 4A to C). Compared with image Figure 4A, the bare gold surface was observed. Figure 4B indicates that modified gold surface was successfully linked to the Au-SAM surface. Figure 4C are aggregates of the bacteriophage over the Au-SAM surface (Shabani et al., 2008; Garcıa-Gonzalez et al., 2008; Chai et al., 2013).

Surface characterization of Gold surface modified electrode using FTIR

Figure 5 confirms the presence of the Functional group over the Gold surface, a fourier Transform Infrared (FTIR) analysis. Characteristic bond found in such as, N-H, C=O and CH_2, stretching vibrations were at 3445.4 cm^{-1}, 1640.4 cm^{-1} and 2926 cm^{-1} wavelength respectively.

Cyclic voltammetry

Cyclic voltammetry is an analytical method used for rapid surface modification and initial characterization. The reversible redox couple that is 1 mM $Fe(CN)_6^{3-/4}$ in deionized water, was selected as a redox probe to study the characteristics of the Au electrode. Figure 6b shows the cyclic voltamograms of the Au electrode in $Fe(CN)_6^{3-/4-}$ solution after different modification steps. $Fe(CN)_6^{3-/4-}$ showed a reversible effect on Au electrode with peak-to-peak separation of 100 mV/s. The self-assembly of the MUA/EDC/NHS and activation of COOH end groups are supplemented by a decrease in the peak current and

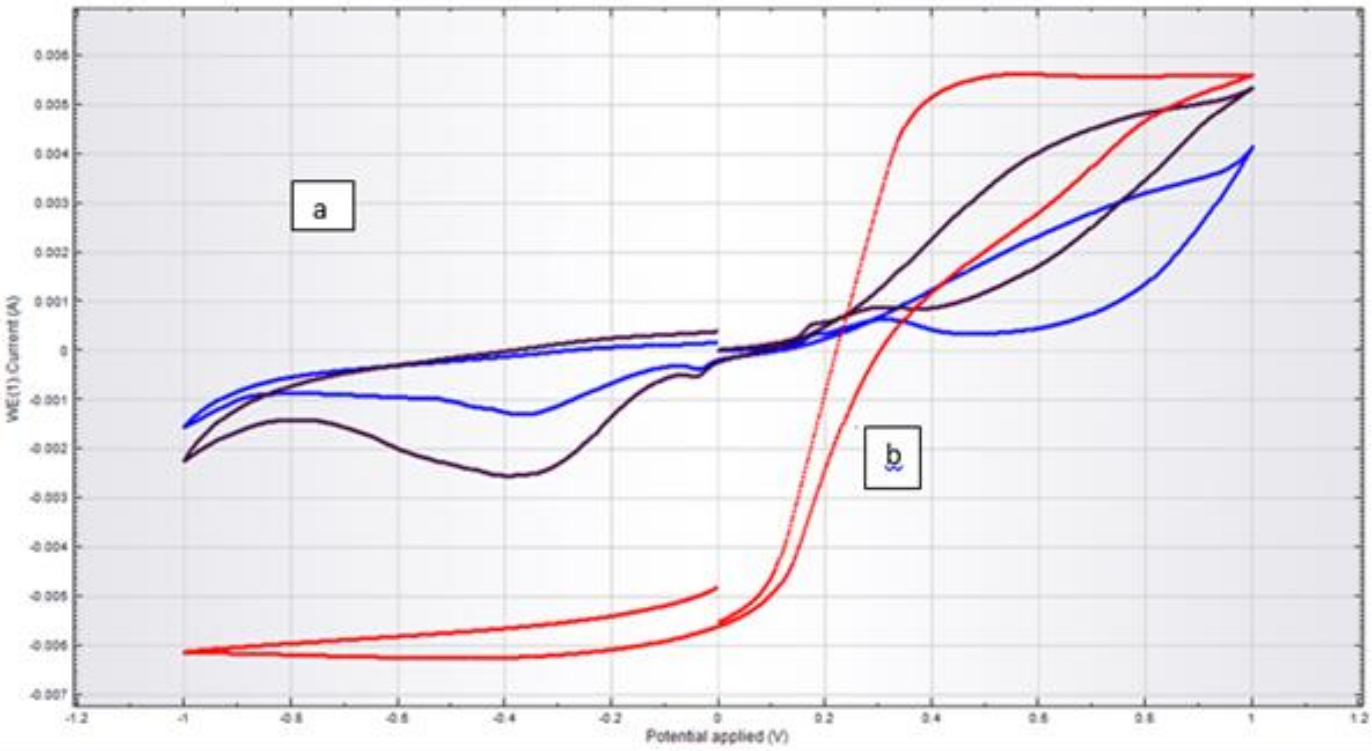

Figure 6. a) Black and Blue curve line are cyclic voltammetry of gold electrode modified with attached phage, different bacterial concentration of *E. coli.* (10^3 and 10^4 cfu/ml) recorded in Phosphate buffer saline. **b)** Red curve line, 1 mm potassium ferric cyanite, as redox active at scan rate of 100 mv/s.

resistance increases due to modification in covering of the electrode at different dilution (10^3 and 10^4 cfu/ml). Figure 6a, depicts the cyclic voltammetry curve of Au working electrode, whose details have been reported in the literature (Lucarelli et al., 2005; Tlili et al., 2013).

Cyclic voltammetry of modified electrode surface scanned at a potential ranging from -1.0 to 1.0 V, at a scan rate of 100mV/s. The formal potential of 5.30x10-3 V was estimated from the mean of the anodic and cathodic peaks of the cyclic voltammogram of the bare Au electrode. Figure 6b shows curve line 1mm potassium ferric cyanite, as redox active at scan rate of 100mV/s. Figure 6a shows the decrease in peak currents can be attributed to the fact that the bacteriophage & MUA/EDC/NHS insulated the surface & effectively altered the electron transfer bariers. One of the reasons being the formation of a barrier by the assembled layer that prevents the access of redox couple. Another might be the low current involved in the redox reaction after functionalization of the Au surface with MUA (Geng et al., 2008).

Electrochemical impedance studies

The impedance spectra displayed a semi-circle and a linear portion. The former at higher frequency represents the electron-transfer limited process and the latter at lower frequency represents the diffusion-limited process (Tlili et al., 2013). Phage modified microelectode in the presence of *E. coli* was placed in PBS solution with the frequency range between 100 MHz-100 KHz (Mejri et al., 2011; Chang Qing et al., 2012). Impedance analysis detection of *E. coli* in apparatus cell, containing sterile PBS, was conducted with different bacterial concentration varying from 10^3 and10^4 CFU/ml. Nyquist plot shows a gradual increase in impedance initially that continued for about 4-15 minutes (Figure 7). The impedance *Z*" (Ω) increased slowly over time until it stabilizes for 10^3 (800) and 10^4 ($3.2x10^3$) cfu/ml, also real impedance Z' (Ω) for both cfu level is 1500 and 3000 increase when compared to bare gold. The semi-circle corresponds to the charge-transfer resistance of the electrode interface (Mejri et al., 2011). The decrease in charge transfer resistance is because of the increase in conductivity after bacteriophage immobilization. This happens because of specific recognition between bacteria and bacteriophage. The subsequent decrease in impedence supposedly occurs as a direct consequence of phage-induced bacteria infection, leading to cell wall disruption and secretion of important amount of intracellular components (Shabani et al., 2008). The medium conductivity in the

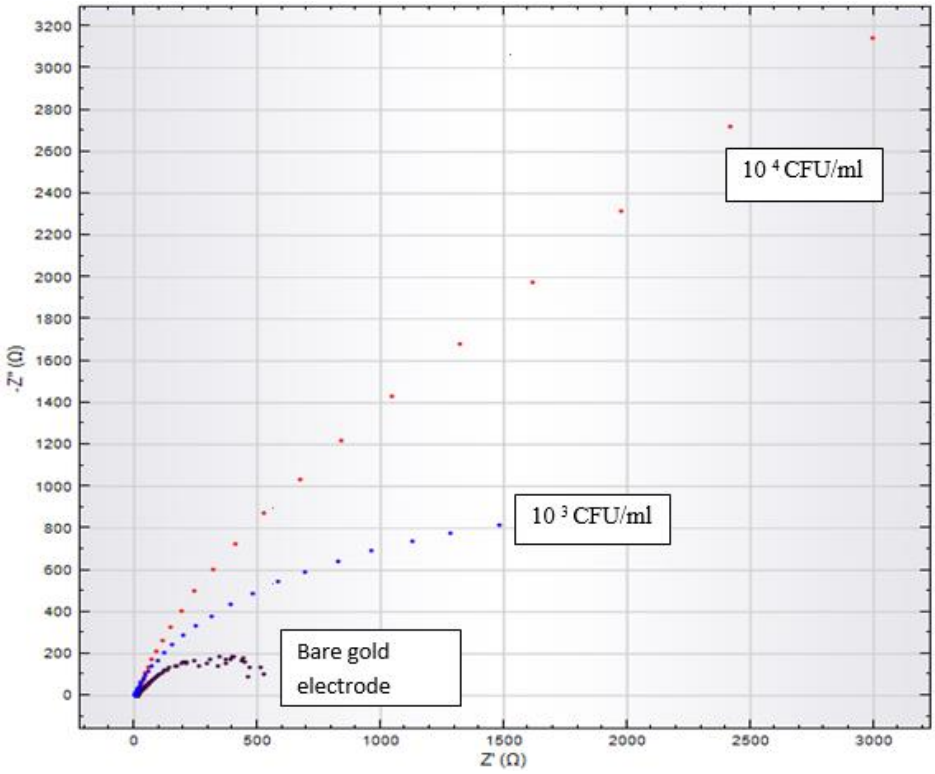

Figure 7. Nyquist plots of impedance spectras obtained for increasing concentrations of e.coli from 10^3 (blue dot curve) cfu/ml and 10^4 cfu/ml (Red dot curve) in phosphate buffered saline (pbs) Au electrode with phage immobilized.

vicinity of the electrode surface is increased as a direct consequence of these events. This increase in medium conductivity decreases the charge transfer resistance (Barreiros dos Santos et al., 2009; Lua et al., 2008).

Conclusion

In the present study, bacteriophage isolated from stagnant waste water sources against *E. coli* and confirmed it to be a lambda like virus after morphological and structural characterization by transmission electron microscopy. Its lytic phage, Plaque reaction activity appear within 4-6 h by spot test method. The bacteriophage then used as a bio component for developing a detection based platform. Isolate phage immobilization was realized through the widely used EDC/NHS cross-linking reaction, which connected the $-NH_2$ group on the surface of phage to the carboxyl end group of MUA self-assembled monolayer on the gold (Au) surface conform by Fourier transformation infrared microscopy presence of functional groups present. Electrochemical measurements including CV and Impedance and Scanning Electron Microscopy observation were carried out to characterize the surface modified gold electrode. The Scanning Electron Microscopy verified that the phage efficiently immobilized onto the Au-MUA/EDC/NHS self-assembled monolayer. CV and Impedance result also showed the electron transfer resistance of the phage-modified electrode. A liner relationship between the electron-transfer resistance and the logarithmic value of *E. coli* concentration was found in the a range of 10^3 and 10^4 cfu/ml with a detection limit of 10^4 cfu/ml. the phage based detection technique introduced here is a real-time, rapid, specific and quantitative method. For future work, generic platform for advanced bacterial sensing, with a high promise in practical applications.

Conflict of interests

The authors did not declare any conflict of interest.

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