

Dendrimer Supported Electrochemical Immunosensor for the Detection of Cholera Toxin in Water

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Generation 2 poly (propylene imine) dendrimer (PPI) and gold nanoparticles (AuNP) were electro-co-deposited on a glassy carbon electrode and used as a platform for the development of an electrochemical immunosensor for the detection of cholera toxin in water. The immunosensor was prepared by the immobilisation of a probe anti-cholera toxin on a PPI-AuNP composite modified carbon electrode for 2 h at 38 °C and subsequently blocked by bovine serum albumin (BSA) to minimize nonspecific binding. Square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) were used for the interrogation of the immunosensor at each stage of preparation. The biosensor was responsive to the toxin over a concentration range of 10^{-7} g mL⁻¹ to 10^{-12} g mL⁻¹ and their SWV and EIS were used to plot the calibration curve with a correlation of 0.9945 for SWV and 0.9966 for EIS. A detection limit of 7.2×10^{-13} g mL⁻¹ and 4.2×10^{-13} g mL⁻¹ were calculated from the square wave voltammetric and electrochemical impedance spectroscopy measurement respectively. The biosensor exhibited good stability over a period of seven days when stored at 4 °C temperature.

Keywords: cholera; poly(propylene imine) dendrimer; cholera toxin; immunosensor; square wave voltammetry; electrochemical impedance spectroscopy.

1. INTRODUCTION

Cholera, a water borne disease caused by cholera toxin or *Vibrio cholerae*, continues to be a major public health concern in developing countries and accounts for considerably high cases of illnesses [1]. Cholera infection results in acute watery diarrhoea with severe dehydration which can lead to death. Since the discovery of cholera in 1883 by the famous German bacteriologist Robert Koch [2], it has continued to be a global menace to date [3]. It is one of the most rapidly spreading

fatal diseases known if no treatment is provide. The severity of cholera epidemics can have a huge social and economic impact on any country. Though the cure for cholera is readily available, the main problem is its sudden outbreaks and fatality (i.e. a lot of infections prior to clinical reports or diagnosis) in areas where treatment is hampered by location or resources. Sub-Saharan Africa, Asia (India and Bangladesh) are among the most affected by cholera. According to Mengel et al “Between 1970 and 2011, African countries reported 3,221,050 suspected cholera cases to the World Health Organization, representing 46% of all cases reported globally. Excluding the Haitian epidemic, sub-Saharan Africa accounted for 86% of reported cases and 99 % of deaths worldwide in 2011” [4]. Countries such as Angola, Democratic Republic of the Congo, Mozambique, Nigeria, Somalia, Tanzania, and South Africa are among the most affected [4]. Cholera outbreaks could have been mitigated if there were faster, cheaper, and on-site cholera detection devices that do not need much expertise to operate. Such a devise will be useful for prompt cholera alert in rural areas were challenges in water sanitation and laboratory facilities are common. There is therefore a need to design cholera biosensors which can be useful on site.

Various methods have been reported for the detection of cholera toxin (CT). Current methods for the detection of cholera toxin are radioimmunoassay [5], enzyme-linked immunosorbent assays (ELISA) [6], and latex agglutination assays [7] and other methods [8]. These methods take several hours to detect CT and are thus incompatible for rapid detection and field screening. The need for fast, sensitive and reliable detection of CT in order to avoid sudden outbreaks underlies the development of cholera biosensors [8-11]. This work seeks to develop an electrochemical immunosensor based on poly (propylene imine) dendrimer-gold platform for the detection of cholera toxin in water.

Dendrimers are synthetic three-dimensional macromolecules with a well-defined, highly branched and globular shaped molecular structure [12]. Poly (propylene imine) dendrimer PPI is biocompatible and has been used in gene delivery as non-viral vectors, in the development of biosensors and for a broad variety of biomedical and industrial applications [13-17]. While PPI is a good candidate for host guest chemistry, the electrodeposited gold nanoparticle [18, 19] on carbon electrodes on the other hand helps in improving electrode conductivity and enhances charge transfer at the biosensor interface [20]. Nanocomposites of PPI dendrimer and gold nanoparticles have been applied as platform for drug delivery systems [21]. PPI-AuNP nanocomposites can offer a suitable platform for immunosensor development.

2. EXPERIMENTAL

2.1 Apparatus and reagents

Generation 2 (G2) poly (propyleneimine) (PPI) dendrimer was purchased from SyMO-Chem, Eindhoven, Netherlands. Cholera toxin B subunit, anti cholera toxin and all other chemicals such as K_2HPO_4 , KH_2PO_4 , KCl , $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, $HAuCl_4$, HCl , $NaOH$ and bovine serum albumin (BSA) were obtained from Sigma Aldrich, South Africa. Ultra-pure water (18.2 M Ω resistivity) prepared by a Millipore Synergy water purification system was used for all the solutions preparation.

100 mM and 10 mM phosphate buffer saline solution (PBS) with pH 7.5 containing K_2HPO_4 , KH_2PO_4 and 0.3 mM KCl was prepared. 5 mM (1:1) solution of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ ($[Fe(CN)_6]^{3-/4-}$) was prepared in 100 mL of 10 mM PBS pH 7.5. 10 mM G2 PPI dendrimer and 5 mM $HAuCl_4$ was used. A 67 mg mL⁻¹ of anti-CT stock was prepared in 0.1 M PBS (pH 7.5) and stored at -20 °C. Working solution was prepared by diluting the stock solution to 0.5 mg mL⁻¹ concentration in PBS, stored at 4 °C. Different concentrations of cholera toxin were prepared by serial dilution from stock solution to make 5 different concentrations from (10⁻⁷ g mL⁻¹ to 10⁻¹² g mL⁻¹), stored at 4 °C. A three electrode cell with glassy carbon, platinum and Ag/AgCl (3 M Cl⁻) as working, auxiliary and reference electrodes respectively was used. All electrochemical experiments were performed on an Autolab PGSTAT 302N using a three electrode system. Square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) were used for analysing the samples.

2.2 Electrode modification

Modification of glassy carbon electrode (GCE) with PPI dendrimer and gold nanoparticles was done according to the procedure reported by Arotiba [17, 22]. Briefly, generation 2 PPI dendrimer and gold nanoparticles (AuNP) was electro-co-deposited onto a glassy carbon electrode from a solution of 10 mM G2 PPI and 5 mM $HAuCl_4$ using cyclic voltammetry. The electrode potential window was cycled from -300 mV to 1000 mV for 10 cycles at a scan rate of 50 mV s⁻¹. The modified electrode was labelled the GCE/PPI-AuNP.

2.3 Immobilisation of probe Anti-CT (antibody)

Immobilisation of anti-CT antibody was prepared on the nano-platform by drop-coating anti-cholera toxin solution on a dendrimer and gold modified GCE surface. The electrode was then left to immobilise for 2 hr at 38 °C to form the immunosensor or simply the biosensor. The immunosensor was rinsed with PBS to remove any unbound probe anti-cholera and named GCE/PPI-AuNP/Anti-CT. The electrode was electrochemically characterised using SWV and EIS in 5 mM $[Fe(CN)_6]^{3-/4-}$ solutions respectively.

2.4 Incubation of cholera toxin (antigen)

For the incubation of cholera toxin antigen, a single concentration of 1.25 x 10⁻⁷ g mL⁻¹ of CT antigen was used. The incubation was prepared by immersing the immunosensor (GCE/PPI-AuNP/Anti-CT) inside the solution of cholera toxin and left it for 1 h at 38 °C to detect the cholera toxin. The electrode was rinsed with PBS and named GCE/PPI-AuNP/Anti-CT/CT. The GCE/PPI-AuNP/Anti-CT/CT electrode was electrochemically characterised using SWV and EIS in 5 mM $[Fe(CN)_6]^{3-/4-}$ supporting electrolyte.

2.5 Immobilisation of anti-CT blocked with (BSA)

The immobilisation of anti-CT blocked with bovine serum albumin (BSA) was prepared in a similar approach as described in section 2.3. The only difference is that, after immobilisation with anti-cholera toxin, the surface of the electrode was blocked with solution of BSA (1 % BSA) for 30 minutes at room temperature. The GCE/PPI-AuNP/Anti-CT/BSA electrode was used to record SWV and EIS. All experiments were done in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4}$ supporting electrolyte. Different concentrations range from (10^{-7} g mL⁻¹ to 10^{-12} g mL⁻¹) of cholera toxin were then prepared by serial dilution to make 5 different concentrations and the SWV and EIS were used to plot the calibration curve.

2.6 Blank binding

Blank experiments were carried out using the GCE/PPI-AuNP/Anti-CT/BSA electrode binding in 0.1 M blank PBS solution (without antigen) in three successive sessions of 30 minutes each at 38 °C. The reason for repeating the successive session was to verify if there will be any changes in the results. The GCE/PPI-AuNP/Anti-CT/BSA electrode was electrochemically characterised using SWV and EIS in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4}$ supporting electrolyte.

2.7 Biosensor Stability Studies

The stability of the biosensor blocked with BSA (GCE/PPI-AuNP/Anti-CT/BSA) was studied for a period of two weeks with SWV and EIS measurements in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4}$ supporting electrolyte. After running the experiments the electrode was stored at 4 °C.

3. RESULTS AND DISCUSSION

3.1 Electrochemical responses of the GCE/PPI-AuNP modified electrode in ferric/ferrocyanide redox probe

The electro-co-deposition of G2 PPI and AuNP onto the glassy carbon electrode surface was accomplished by cycling between the potential window of -300 mV to 1000 mV for 10 cycles at a scan rate of 50 mV s⁻¹. As discussed by Arotiba et al [17, 22] the free primary amines of the PPI were electro-oxidised onto the GCE surface to form C-N bond in the anodic scan, while the cathodic scan was used to deposit the gold nanoparticle by reducing the gold from Au³⁺ to Au⁰. Fig. 1 presents the SWV and EIS interrogations of the biosensor preparation steps. As shown in figure 1a, the presence of the dendrimer-gold nanocomposite enhanced the faradaic current generated during the redox reaction of the ferro/ferric cyanide redox probe. This phenomenon can be related to the enhanced surface area and probably electrocatalysis at the interface of the nanocomposite. The impedance plot supports the enhanced current observed in the square wave voltammetry. The dendrimer – gold modifier enhanced

electron transfer at the electrode interface which in turn reduced the charge transfer resistance of the system.

3.2 Immobilisation of probe Anti-CT

After the preparation of the modified electrode (GCE/PPI-AuNP), the probe anti-cholera toxin was immobilised on the GCE/PPI-AuNP platform. The use of the dendrimer-gold nanocomposite as platform is based on the following reasons: 1) PPI has been shown to have some elements of biocompatibility and will thus favour the adsorption of the anti-cholera toxin [12]. 2) At neutral pH, the difference in the charge (PPI is positive while the anti-cholera toxin is negative) of the platform and the bioreceptor is exploited for supramolecular attraction (electrostatic attraction) and not just mere physical adsorption. 3) We believe that the dendrimer has the capability of forming a host-guest interaction with the bioreceptor owing to its nano-voids which can further entrap the cholera toxin. The peak current of the redox probe decreased after the antibody immobilisation of the anti-cholera bioreceptor (Fig. 1a). The reason for the decrease in current is because the poor conductivity of the antibody hampered the electroactivity of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at the electrode interface. The current reduction confirmed that the anti-CT was successfully attached to the surface of the electrode. The anti-cholera toxin immobilised electrode is referred to as the GCE/PPI-AuNP/Anti-CT or the biosensor. EIS was also used to characterise the GCE/PPI-AuNP/Anti-CT electrode in $\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe. EIS (Fig 1b) was carried out at 10 mV AC potential and 200 mV dc potential. The impedance data was fitted using the common Randles circuit model consisting of solution resistance R_s , in series with a parallel combination of charge transfer resistances R_{ct} and a double layer capacitance (C_{dl}) with a Warburg impedance (Z_w). The charge transfer resistance increased when the Anti-CT probe was immobilised on the surface of the modified PPI-AuNP electrode (increased from $R_{ct} = 34.2 \Omega$ modified to $R_{ct} = 447 \Omega$ immobilisation). The increase in charge transfer resistance can be attributed to the poor conductivity of the antibody which hindered the electron transfer of the redox probe at the GCE/PPI-AuNP/Anti-CT - $\text{Fe}(\text{CN})_6]^{3-/4-}$ interface. The change in R_{ct} value was used as an indication of successful immobilisation.

3.3 Immobilisation of anti-CT blocked with BSA

The immobilisation of anti-CT blocked with BSA was studied in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as a supporting electrolyte. Usually when a bioreceptor such as cholera antibody is immobilised on an electrode, there still remain some unbound sites on the electrode surface. These unbound sites can cause non-specific adsorption. That is, the target (cholera toxin in this case), attaches itself to the free sites (GCE/PPIAuNP) instead of binding to the probe (antibody) causing a false result. In order to overcome this non-specific binding, blocking is usually carried out. A molecule such as BSA, capable of adsorbing on the unbound site and unreactive towards the target is commonly used [9, 23]. The blocking process was carried out on the surface of the anti-cholera toxin electrode by drop coating with a solution of BSA for 30 minutes at room temperature. SWV and EIS were used to monitor the

blocking step. The SWV result showed the reduced peak current in comparison to the biosensor (Fig 1a). This is expected because the BSA is not electroactive and thus further insulates the electrode surface by blocking the free sites available for electron transfer.

A similar observation was recorded for the EIS experiment (figure 1b). After blocking with BSA, the charge transfer resistance slightly increased. The increase in the charge transfer resistance value shows that the BSA indeed blocked the unbound surfaces.

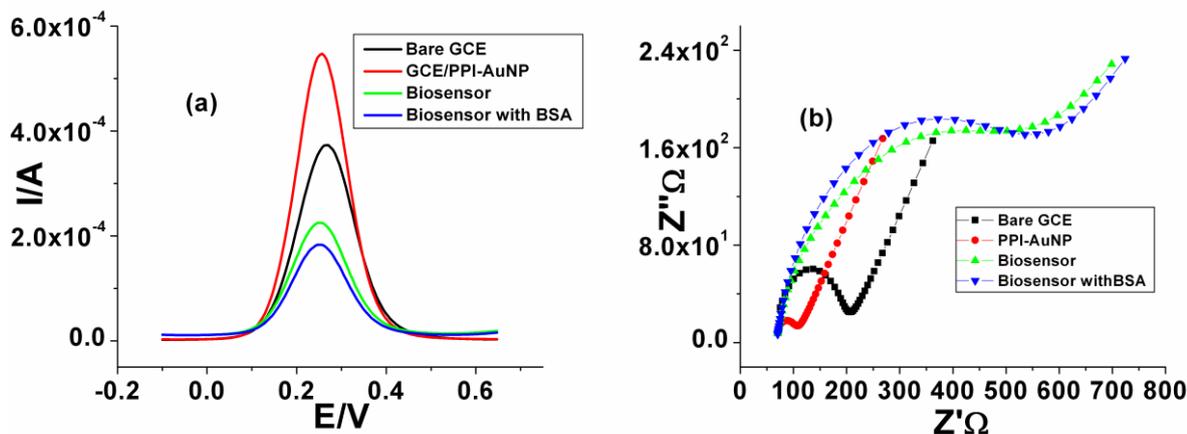
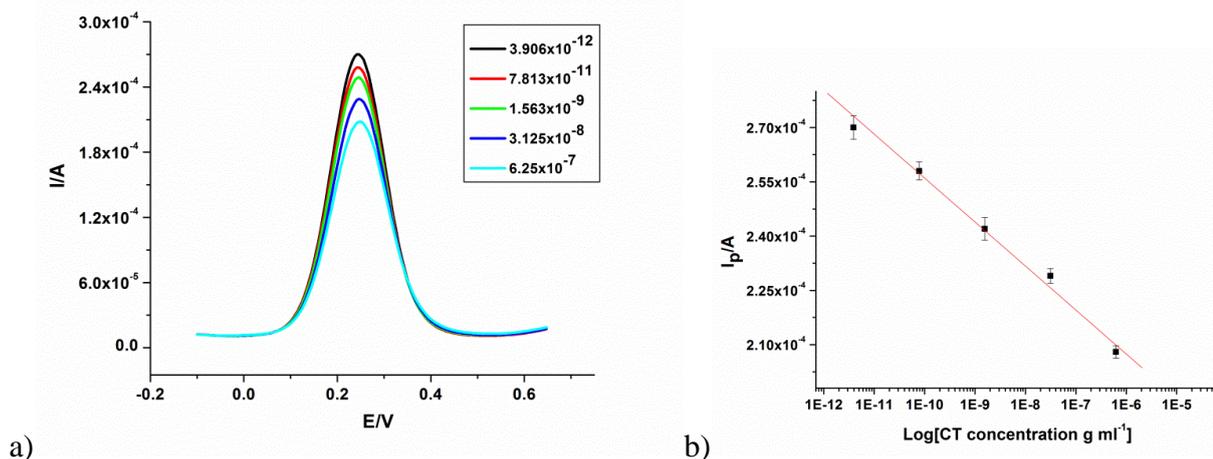


Figure 1. Electrochemical interrogation of the biosensor preparation steps in the presence of 5 mM $[\text{Fe}(\text{CN})_6]^{3/4-}$ redox probe and the effect of blocking on the biosensor. (a) SWV at 25 Hz. (b) EIS.

3.4 Biorecognition of the CT target and calibration curve

SWV and EIS were then used to record the signal generated by the biorecognition event between the biosensor (the attached anti cholera toxin) and the target (cholera toxin) at different concentrations in 5 mM $[\text{Fe}(\text{CN})_6]^{3/4-}$ supporting electrolyte and respective calibration graph were plotted (Fig 2).



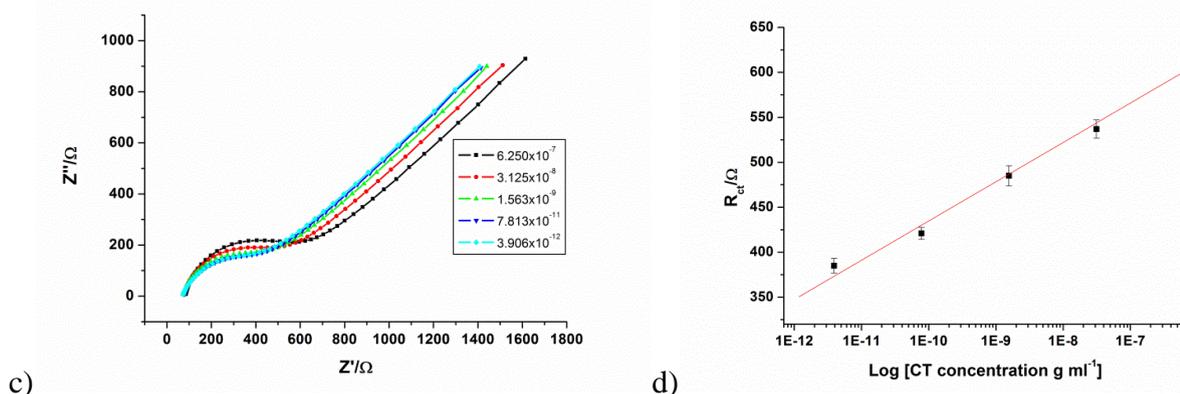


Figure 2. Biosensor response to different concentrations of CT target in 5 mM $[\text{Fe}(\text{CN})_6]^{-3/4}$: a) SWV, b) SWV calibration plot (3 replicates), c) EIS, d) EIS calibration plot (3 replicates).

The calibration plot of peak anodic current (I_{pa}) versus log of concentration showed the linearity with correlation coefficient of 0.9945 (Fig. 2a and b). The biosensor was responsive to the toxin over a concentration range of 10^{-7} g mL $^{-1}$ to 10^{-12} g mL $^{-1}$. EIS was also used for the detection of cholera toxin at different concentrations in 5 mM $[\text{Fe}(\text{CN})_6]^{-3/4}$ supporting electrolyte (Fig. 2c). The calibration graph of cholera detection was plotted (Fig. 2d). The biosensor was linear over the same range as that of SWV with a correlation coefficient of 0.9966. This biosensor can thus be both voltammetric and impedimetric.

3.5 Blank binding and stability

The biosensor was subjected to blank incubation. That is, a target solution containing 0.1 M phosphate buffer saline solution void of CT. The blank solution was deposited under the same conditions as the CT target and measurements were taken in three successions of 30 minutes interval. The SWV and EIS data showed no appreciable differences after binding, as shown in Fig. 3a and b. These results denote that the biosensor responses to CT target were as a result of the bio-recognition event and not just due to mere solvent or electrolyte interaction at the electrode interface. The detection limit of the biosensor was calculated using the formula $\frac{3 \times \text{SD}}{\text{slope}}$, where SD is the standard deviation of the blank measurements and the slope of the calibration curve. A detection limit of 7.2×10^{-13} g mL $^{-1}$ and 4.2×10^{-13} g mL $^{-1}$ were calculated from the square wave and electrochemical impedance spectroscopy measurement respectively. The similarity between these two values suggests that the two methods are in agreement. The linear detection range and detection limits reported in this work compares well with those reported in the literature [10, 24]. The minimum amount of toxin in water for cholera to occur is not yet standardised though a range of ng mL $^{-1}$ has been suggested [6]. The detection limit obtained in this report is however comparable to that obtained by Baemner and co-workers [9] and Chiriaco et al

[25] and other reports cited earlier in this paper. The unstandardized toxin level may be due to the fact that as long as the bacteria are present and thriving, there is likelihood of increased toxin production.

The biosensor (already blocked with BSA) GCE/PPI-AuNP/Anti-CT/BSA was kept at 4 °C when not in use. Stability of the biosensor was studied over 14 days by measuring the SWV current signal in the redox probe (Fig 3c). The biosensor exhibited good stability in the first seven days. However, a noticeable reduction of ca 12% in the voltammetric current was observed day 14.

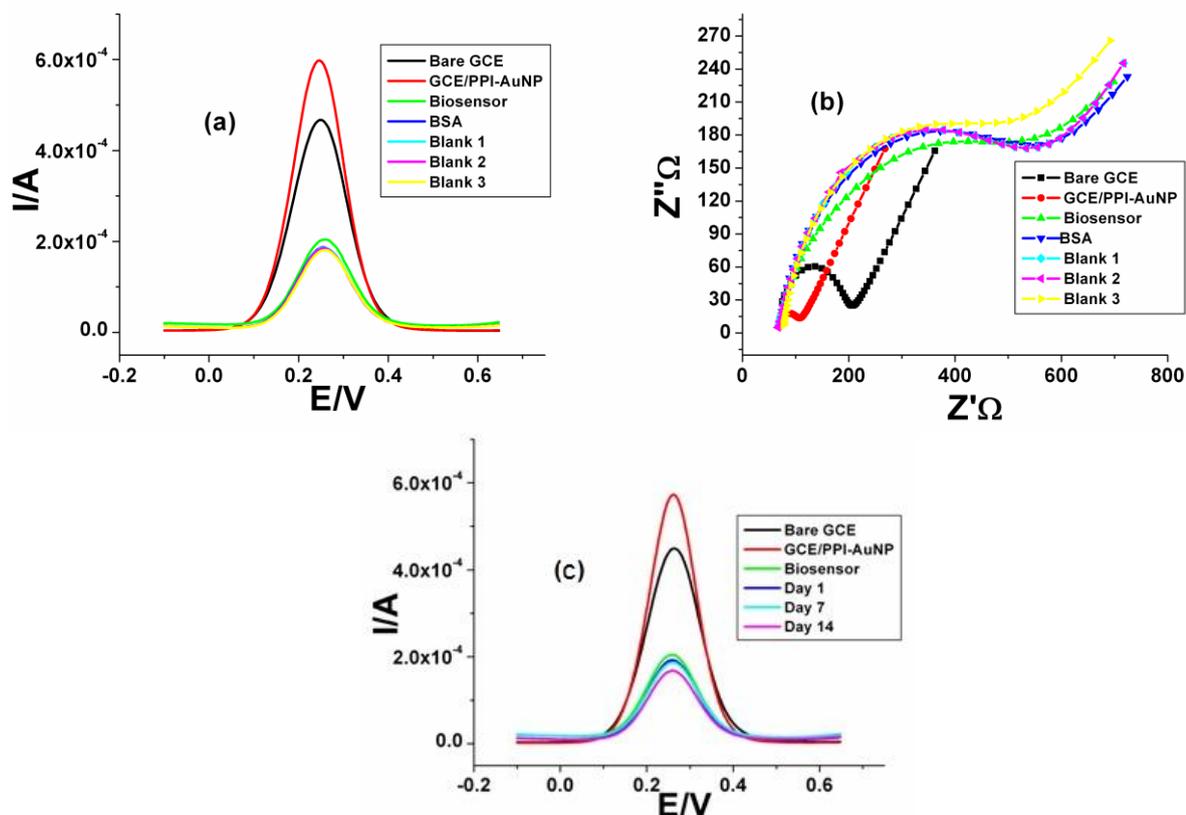


Figure 3. (a) SWV in 5 mM [Fe(CN)₆]^{3-/4-} blank binding (b) EIS in 5 mM [Fe(CN)₆]^{3-/4-} blank binding (c) SWV responses of the biosensor in 5 mM [Fe(CN)₆]^{3-/4-} at different days.

4. CONCLUSIONS

This work demonstrates a novel application of dendrimer-gold particle composite in the development of an immunosensor for a very important communicable disease - cholera. The versatility of PPI dendrimer as a biocompatible layer for the immobilisation of biomolecules and possible biomedical applications has again been demonstrated. The biosensor was able to qualitatively and quantitatively detect cholera toxin in standard solutions. The composite platform showed good electrochemical properties suitable for biosensor development. The biosensor performance can be monitored with two different electrochemical techniques – electrochemical impedance spectroscopy

and square wave voltammetry. This dual approach is a merit to the developed biosensor. The biosensor demonstrated good stability for up to two weeks.

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