A Voltammetric Immunosensor for Detection of HER2 using Gold Modified-Screen Printed Carbon Electrode

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Abstract

HER2 (human epidermal growth factor receptor 2) is referred as a prognostic marker key, so the determination of HER2 concentration is key to the diagnosis and treatment of breast cancer. This research aims to develop a label-free voltammetric immunosensor detection of HER2. The SPCE (screen printed carbon electrode) is modified with chloroauric acid electrodeposition at potential from -1.5 to 0.6 V for 10 cycles. Anti-HER2 was immobilized on the gold-modified SPCE based on amine group covalent bonds with gold surfaces, and the attachment of HER2 is characterized by a decrease in peak current of potassium ferricyanide.

The results of this study show that peak response of reduction-oxidation current of potassium ferricyanide on cyclic voltammogram on SPCE without and with gold modification with increase of 39.9 percent and after anti-HER2 immobilization showed a decrease of 28.5 percent and sensitivity 0.2906 for a concentration range of 0 to 10 ng/mL, limit of detection 2.9 ng/mL, limit of quantification 8.4 ng/mL, 89.7 percent of precision and 99.1 percent of accuracy. This electrochemical immunosensor can be applied to detect HER2 for early diagnosis of breast cancer.

Keywords: Voltammetric immunosensor, HER2, anti-HER2, gold-SPCE

Introduction

HER2 is a receptor tyrosine kinase which belongs to the epidermal growth factor receptor (EGFR) family involved in cellular signaling pathways that may lead to proliferation, growth, apoptosis and differentiation[1]. Over expression of HER2 is found in various cancers such as breast, ovarian, stomach, lung adenocarcinoma, endometrial cancer, gastric cancer and salivary gland cancer, but more studied in breast cancer with over expression of 25-30 percent. Normal breasts have about 20,000 HER2 receptors whereas in breast cancers they multiply to about 1.5 million HER2 receptors on the cell surface[2]. HER2 is referred to as a prognostic marker key, so the determination of HER2 concentration is key to the diagnosis and treatment of breast cancer[3].

There are several methods for detecting HER2 positive cells that have been developed including fluorescent in situ hybridization that involve tumor cell analysis for HER2 gene amplification and immunohistochemistry to determine the expression of receptors in cell membranes[1], colorimetry[4], fluorescence[5], scattering assays[6] and cell imaging[7]. Most of these techniques are limited to cell detection and are not available for measuring the concentration of serum HER2 which offers a far less invasive method of determining the HER2 status than a biopsy[8]. Some are complicated, time consuming and require specially trained personnel to carry out the corresponding multi-step procedures[9].

Recently, electrochemical sensors have been considered as valid alternatives to classical analysis methods for detection of cancer markers and widely used in point-of-care devices due to the advantages of being portable, simple, easy to use, robust, cost-effective, disposable and capable of multi-analyte testing[10,11].

Several electrochemical immunosensors have been proposed for the detection of HER2. An electrochemical immunosensor has been developed by immobilizing streptavidin-biotin monoclonal antibody HER2 on SPCE based on sandwich ELISA (Enzyme-Linked Immuno Sorbent Assay) that labeled alkaline phosphatase secondary antibody used as trastuzumab on gold nanodisk electrodes. The interaction between HER2 and antibody is detected by suitable secondary antibodies labeled with HRP (horseradish peroxidase) and methylene blue as the redox mediator. This immunosensor is used to determine HER2 in cell lysates and tumor lysates. The limit of detection (LOD) was found approximately as 0.1 ng/µL[12].

Another electrochemical immunosensor has been developed based on a sandwich format in which a primary monoclonal antibody anti-HER2 is coupled to protein a-modified magnetic beads to capture HER2. The sandwich assay is performed by adding a secondary biotinylated polyclonal antibody anti-HER2. Streptavidin-alkaline phosphatase conjugated was then added to trace the affinity reaction. The enzymatic substrate, naphthyl-phosphate, is then used for the electrochemical detection by voltammetry technique with the LOD of 6 ng mL[1,9]. A label free platform for detection of HER2 based on antiHER2-iron oxide nanoparticle bioconjugates has been published. After the bioconjugates are immobilized over the gold electrode surface, the measurement was done using differential pulse voltammetry technique and a LOD of 0.995 pg/mL were found[14].

Patris et al[15] have developed a sandwich type immunoassay (nano-immunoassay) using Nanobodies (Nbs) linked to a carbon-based screen-printed electrode (SPE) for the HER2 extracellular domain determination with a LOD of 1.0 µg/mL. An electrochemical immunosensor for the

quantification of HER2 ECD in serum was developed by using a gold nanoparticle-modified screen-printed carbon electrode. Monoclonal anti-human-HER2 antibodies were immobilized on the transducer surface and after incubation with a HER2 ECD containing sample biotinylated monoclonal anti-human-HER2, detection antibodies were added. A streptavidin–alkaline phosphatase conjugate was used to label the detection antibody.

The detection of the antibody–antigen interaction was possible by using an enzymatic substrate (3-indoxyl phosphate, 3-IP) and silver ions that can be analyzed by anodic stripping using linear sweep voltammetry. The LOD was found as 6.0 ng/mL[16]. An impedimetric immunosensor based on a gold nanoparticles/ multiwall carbon nanotube-ionic liquid electrode (AuNPs/MW-CILE) was developed for the determination of HER2. The results showed that the charge transfer resistance increases linearly with increasing concentrations of HER2 antigen. The LOD was found as 7.4 ng/mL[11].

In this research, we proposed a simple label free electrochemical immunosensor for HER2 detection by using a gold modified via chloroauric acid electrodeposition on screen printed carbon electrode as transducer. Monoclonal anti-HER2 antibodies were immobilized on the transducer surface via mercaptopropionic acid sorption and ethyl-dimethylaminopropyl carbodiimide/hydroxyl succinimide, and then were incubated with a HER2. The detection of the antibody–antigen interaction was possible by using a decrease of reduction-oxidation of potassium ferricyanide that can be analyzed by cyclic voltammetry.

Material and Methods

Materials: Trastuzumab (Roche), HER2, hydrochloric acid (HCI), chloroauric acid ($HAuCl_4$), 3-mercaptopropionic acid (MPA), N-ethyl-N'-[3-dimethylaminopropyl] carbodiimide (EDC), N-hydroxysuccinimide (NHS) and ethanolamine (Sigma), potassium chloride, sodium hydroxide, sodium chloride, sodium hydrogen phosphate, potassium dihydrogen phosphate, potassium ferricyanide, potassium chloride, ethanol (Merck) and Milli-Q water (inhouse lab).

Anti-HER2 immobilization on SPCE and characterization of modified electrochemical electrode: SPCE consisting of working electrode made of carbon, carbon-based auxiliary electrode, and Ag/AgCl as a reference electrode, was dripped with a 40 µL of chloroauric acid 1000 µg/mL solution and electrodeposited with differential pulse voltammetry with a potential range from -1.5 to 0.6 V in 10 cycles at a scan rate of 0.1 V/s[5], then rinsed carefully with Milli-Q water and dried in the air. The SPCE modified gold nanoparticles are electrochemically characterized by cyclic voltammetry with a potential range of -0.6 to 0.6 V with a scan rate of 0.05 V/s by dipping in a 10 mM of potassium ferricyanide solution in potassium chloride 0.1 M. SPCE before and after modification was also characterized using SEM.

The SPCE has been modified with gold nanoparticles, incubated in 0.01 M of MPA solution in ethanol for two hours at 25˚C to form SPCE/GNP/MPA, then rinsed with ethanol. The gold nanoparticles were electrodeposited in SPCE and then incubated with MPA because MPA is an alkanethiol organic molecule that can be strongly and spontaneously chemisorbed onto the gold surface. The electrode was rinsed with ethanol in order to remove the physically adsorbed thiol. Then as much as 30 µL of EDC/NHS solution (0.1 M/0.1 M) was dripped on the SPCE surface and incubated for one hour at 25˚C and then rinsed with Milli-Q water.

The addition of EDC/NHS was performed to activate the carboxylic acid group in the MPA on the electrode surface to give the reactive succinimide ester (figure 1). Then the electrode was dripped with anti-HER2 (5 µg/mL of 30 µL) and incubated for one hour at 25˚C to obtain the SPCE/GNP/MPA/Ab electrode. Reactive succinimide ester reacts spontaneously with the primary amine group present in the antibody and then rinsed with PBS pH 7.4. After that, the electrode was dripped with 5 µg/mL of anti-HER2 and incubated for one hour at 25°C to obtain the SPCE/GNP/MPA/Ab electrode. Then the electrode was rinsed with PBS pH 7.4. After that, the electrode was dripped with 30 µL of ethanolamine 1 M and incubated for 20 minutes and then rinsed with Milli-Q water.

The addition of ethanolamine was performed to deactivate the remaining ester. After that, the electrode was dripped with 30 µL of HER2 and incubated for 30 minutes at 25°C to obtain the SPCE/GNP/MPA/Ab/Ag and then rinsed with PBS pH 7.4[11,17]. To determine whether MPA, anti-HER2 and HER2 have been successfully immobilized, an electrochemical measurement method is performed. SPCE before modification and after modification with the aim of immobilization on the addition of MPA, anti-HER2 and HER2, was measured by cyclic voltammetry using redox system of potassium ferricyanide solution 10 mM in potassium chloride 0.1 M in potential range -0.6 V to 0.6 V with a scan rate of 0.05 V/s, and then rinsed with Milli-Q water.

Results and Discussion

Characterization results of SPCE with gold modification using SEM: The immunosensor response is related to its morphology. The SEM results of a) SPCE (screen printed carbon electrode) and b) SPCE/GNP (screen printed carbon electrode/gold nanoparticle) are shown in figure 2. SEM characterization of SPCE shows a uniform surface whereas SEM characterization of SPCE after modification shows distribution of nano-sized gold electrodeposits.

Characterization results of SPCE with gold modification using Cyclic Voltammetry: The SPCE response before and after the gold modified via electrodeposition of chloroauric acid solution was electrochemically observed using cyclic

voltammetry in potassium ferricyanide 10 mM in a 0.1 M potassium chloride solution (figure 3).

Figure 3 shows that electrodeposition of chloroauric acid solution on SPCE increases the current response of ferricyanide/ferrocyanide. This is because gold increases the conductivity of the electrode by increasing the electron transfer between the electrode and the analyte.

Immobilization of Anti-HER2 on SPCE and electrochemical characterization of modified electrode: In this study, a label-free immunosensor was used so that all electrochemical measurements were performed by observing the redox activity of the electroactive species ferricyanide/ferrocyanide on the electrode surface. Ferricyanide was selected as mediator to check the electrode changes before and after each modification process. The reaction of the redox pair system ferricyanide/ferrocyanide is as follows:

$$[Fe(CN)_6]^{3-} + e \leftrightarrows [Fe(CN)_6]^{4-}$$

When the immunoreaction occurs at the modified electrode, the non-conductive immunocomplex as if it is an isolator, increases the resistance to inhibit electron transfer directly from the redox electron mediator, resulting in a decrease in the electrochemical signal. Therefore, revenue signals from mediators are essential in immunosensors without this marker.

Figure 4 shows the cyclic voltammogram of a carbon dipped in potassium ferricyanide 10 mM electrode in 0.1 M potassium chloride solution. As shown in the figure, the redox system of ferricyanide/ferrocyanide in SPCE (curve a) and GNP (curve b) shows an increase in peak current of 39.9% after SPCE modification with gold nanoparticles (GNP).

This is due to the presence of GNP electrodeposition on SPCE that provides an effective surface with increased conductivity derived from gold nanoparticles, compared to carbon-only electrodes. In addition, the presence of gold nanoparticles also provides conduction path requirements for acting like nanoscale electrodes by promoting electron transfer between the analyte and the electrode surface.

Subsequently, MPA (curve c) was added to the electrode surface to form SPCE/GNP/MPA, a decrease in peak current from measured peak current is shown. This suggests that MPA has been chemisorbed in gold nanoparticles since the presence of MPA on the electrode surface causes the obstruction of electron transfer between ferri-cyanide/ferrocyanide and electrode caused by the compactness on the electrode surface after the occurrence of a negligible MPA and gold nanoparticles on the surface of the electrode. Thereafter, the decrease in peak current response occurs again when anti-HER2 immobilization (curve d) via EDC/NHS forms SPCE/GNP/MPA/Ab. This suggests that immobilized anti-HER2 is causing the ferricyanide/ ferrocyanide species further from the electrode surface where the electron transfer is increasingly obstructed, in accordance with the decreased conductivity of the modified electrode. The decrease in peak current response was compared after modification of the gold nanoparticles by 28.5 percent.

Then, the peak current response decrease occurs again when HER2 was added to the electrode surface to form SPCE/GNP/MPA/Ab/Ag (curve e). This suggests an interaction between HER2 and anti-HER2 as a form of antibody-antigen linkage and as an inert electron transfer layer barrier at the surface of the electrode that can widely avoid the diffusion of the redox pairs to the electrode surface.

Current response to MPA concentration: The influence of the concentration of MPA (3-mercaptopropionic acid) to the anti-HER2 immobilization efficiency was tested using cyclic voltammetry with a potential range of -0.6 to 0.6 V at a scan rate of 0.05 V/s in a solution of potassium ferricyanide 10 mM containing potassium chloride 0.1 M. MPA is essential in the development of immunosensors because of carboxylic groups of MPA that react with the primary amine group of antibodies. The SPCE has been modified with gold nanoparticles, incubated in a solution of MPA variations in concentrations of 0.1 and 0.01 M for 12 hours.

The measurement results show that MPA concentration of 0.1 M resulted in a lower current than the MPA concentration of 0.01 M. However, MPA with a concentration of 0.1 M can cause cracks in the ceramic in SPCE due to high concentration of MPA. As this could interfere with the immunosensor analysis process, the selected concentration is 0.01 M.

Current response to MPA incubation time: The influence of incubation time MPA (3-mercaptopropionic acid) to the anti-HER2 immobilization efficiency was tested using cyclic voltammetry with a potential range of -0.6 to 0.6 V at a scan rate of 0.05 V/s in a solution of potassium ferricyanide 10 mM containing potassium chloride 0.1 M. MPA is essential in the development of immunosensors because of carboxylic groups of MPA that react with the primary amine group of antibodies. The SPCE has modified gold nanoparticles incubated in MPA 0.01 M solution for 1, 2, 12, and 16 hours.

The results of the measurements show that the longer is the MPA incubation time, the greater is the current decrease as more MPA is chemisorbed by gold nanoparticles on the electrode. At 16 hours incubation time the lowest current was produced, but 16 hours time is considered too long for the analysis process. Therefore, the possible incubation time is between 2 and 12 hours. However, as incubation time of 2 and 12 hours produces almost the same current, 2 hours incubation time is chosen as incubation time for MPA.

Current response to optimum concentration of anti-HER2: Anti-HER2 effect of concentration on the efficiency of immobilization was tested using cyclic voltammetry with a potential range of -0.6 to 0.6 V at a scan rate of 0.05 V/s in a solution of potassium ferricyanide 10 mM containing potassium chloride 0.1 M. Anti-HER2 is essential in the development of immunosensors as it relates to the sensitivity of immunosensor detection to HER2. Anti-HER2 concentration variations of 50, 10, 5, 1 and 0.1 μg/mL were tested on this optimization.

The measurement results show that there is a decrease in current with the increase of antibody concentration from 0.1 μg/mL up to 5 μg/mL. A possible reason is that the antibodies attached on the gold nanoparticles through linker (EDC/NHS) with suitable orientation caused the improved orientation of immobilized antibodies to participate in immunoreactions.

However, too low anti-HER2 concentration will result in lower currents as well because the immobilized antibodies are too low and the recognition side to HER2 is reduced. In this case, higher concentrations of antibodies might increase the current with the increased amounts of antibodies from a concentration of 5 μg/mL to reach 50 μg/mL. A possible reason is that larger amounts of antibodies can increase the disorder in alignment of the attached antibody and may result in steric hindrance of immunoreaction because the antibodies immobilized on the surface of the electrode are already saturated, therefore disrupting the process of immobilization, resulting in poorer orientation immobilization and reducing the amount of anti-HER2 antibody that reacted to MPA.

The density or the number of antibodies immobilized on the electrode surface should also not be too high as it may cause steric hindrances between antibodies at the electrode that will reduce the recognition activity. Thus, the concentration of 5 μg/mL is chosen as the optimum concentration for the prepared sensor.

Calibration curve: In the preparation of calibration curves for the voltammetric immunosensor method using a gold-modified SPCE, use HER2 with concentration variations of 0, 0.005, 0.01, 0.015, 0.02, 0.1, 1 and 10 ng/mL. Then each concentration was measured to obtain a standard deviation measurement value which is useful for further data analysis. The results of measurement of HER2 antigen concentration variation are shown in figure 5.

As seen in figure 5, there is a decrease in peak current along with increasing HER2 concentrations from 0 to 10 ng/mL. This shows that there is an inverse relationship between HER2 concentration and peak current response. The relationship is then plotted into a calibration curve of the variation of HER2 concentration to the peak current response shown in figure 6. As shown in figure 6, there is a straight-line relationship between the HER2 concentrations with the peak current. The linear regression equation for the above calibration curve is $y = -0.2906x + 4.4282$ with the value of $R^2 = 0.9783$.

The peak current response resulting from the concentration of HER2 of 0, 0.005, 0.01, 0.015, 0.02, 0.1, 1 and 10 ng/mL was tested statistically to determine whether the concentration is within the linearity range of the calibration curve. From the test results, it is known that there is a decrease in peak current along with the increase of HER2 concentration from 0 to 10 ng/mL, with the value of regression coefficient being -0.9936. The value is included in the range $-1 \leq r \leq 1$. The negative sign on the value of the regression coefficient denotes the effect of variable x to variable y where the correlation between the two variables is opposite which means an increase in the value of variable x (concentration) will be accompanied by a decrease in value on variable y (current). As such it can be concluded that the concentration range of 0 to 10 ng/mL is within the test range linearity calibration curve.

Sensitivity testing was performed to determine the concentration that can be reached by immunosensor. The sensitivity of the voltammetric immunosensor method is expressed by the slope value of the linear regression equation of the calibration curve i.e. -0.2906 which means that for each concentration increase of 1, the average current is reduced by 0.2903 μA.

Determination of the limit of detection used the equation $y = yb + 3\ Sb$, then obtain the limit of detection value for the measurement of 2.9 ng/mL. The limit of detection is still smaller than the limit of detection of a study conducted by Arkan et al[11] and Marques et al[16] which is 7.2 ng/mL and 4.4 ng/mL but is larger than the research conducted by Emami et al[14] which is 0.995 pg/mL. This may be due to the research of Emami et al[14] which uses bioconjugate at the electrode which further enhances the sensitivity of the immunosensor. However, these developed immunosensors are simpler, faster, sensitive, require less reagents and can be used to detect HER2.

In the determination of the limit of quantification, the equation $y = yb + 10\ Sb$ was used and obtain the limit of quantification value of the measurement of 8.4 ng/mL. The determination of precision and accuracy is done by testing one of the concentrations of HER2 in the linear range of the calibration curve. The peak current response generated at the measurement of concentration of 10 ng/mL was incorporated into the equation $y = -0.2906x + 4.4282$. Precision states the proximity of values of two or more measurements by the same method whereas the accuracy states the proximity of the average value of measurement to the true value. Based on the calculation, obtain precision value measurement result which was 10 ng/mL that is 89.7 percent and accuracy value that is 99.1 per cent.

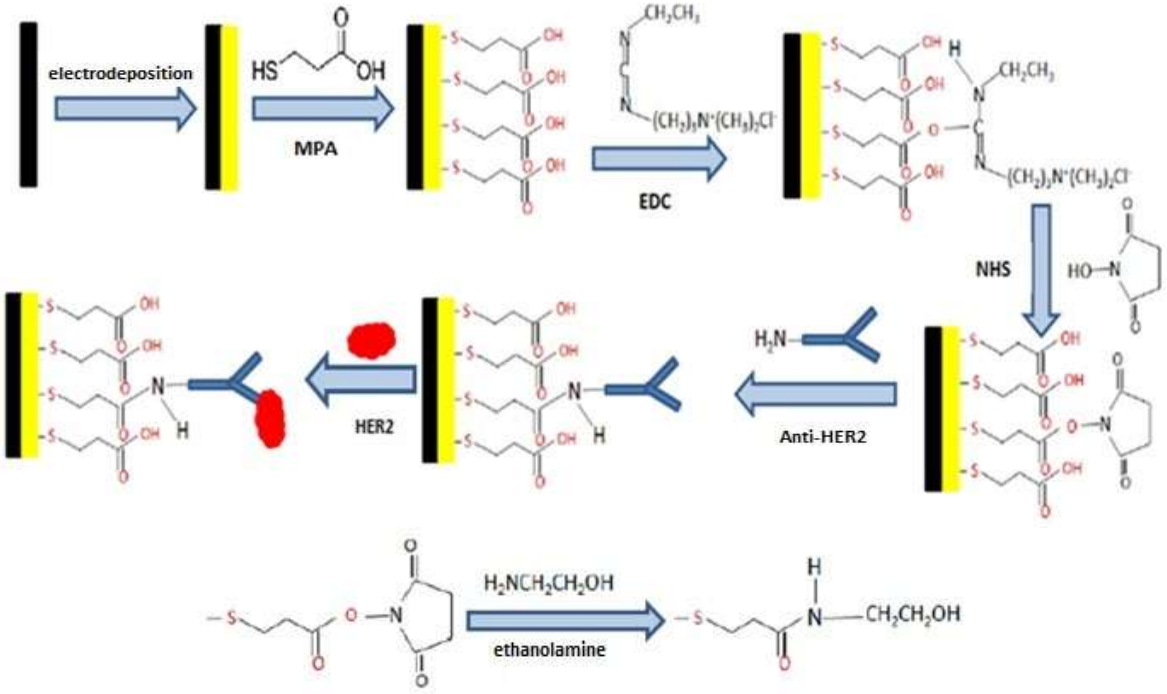

Figure 1: Schematic of immunosensor stage

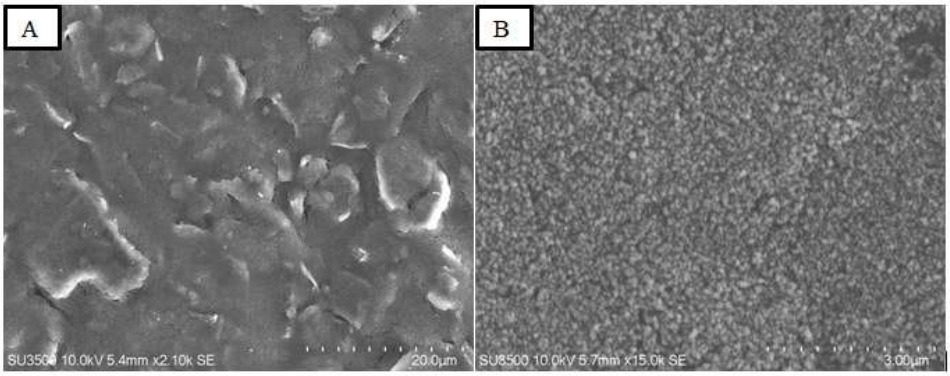

Figure 2: SEM Patterns of a) SPCE; b) SPCE/GNP

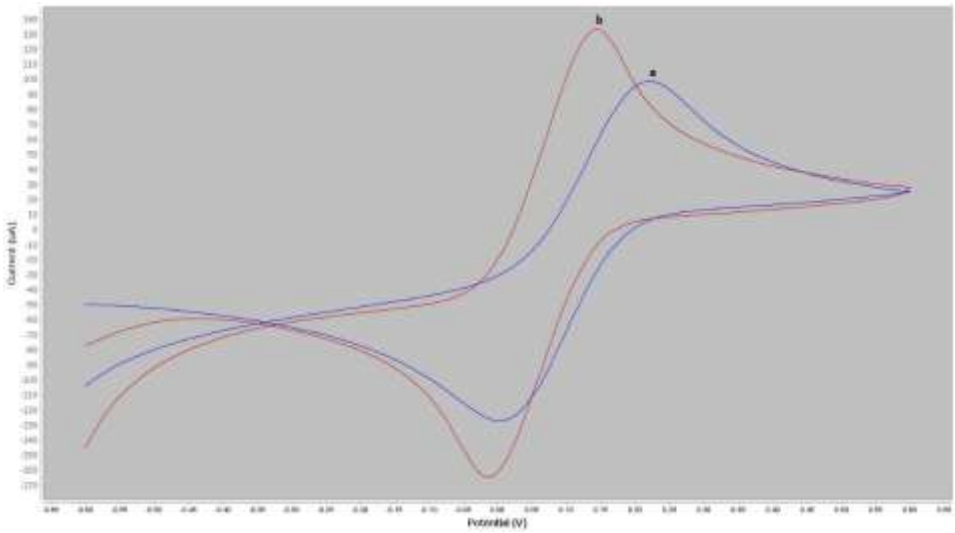

Figure 3: Cyclic voltammogram of electrochemical characterization: a) SPCE; b) SPCE/GNP. In a solution of potassium ferricyanide 10 mM containing potassium chloride 0.1 M. Scan rate was 0.05 V/s in the potential range -0.6 V to 0.6 V.

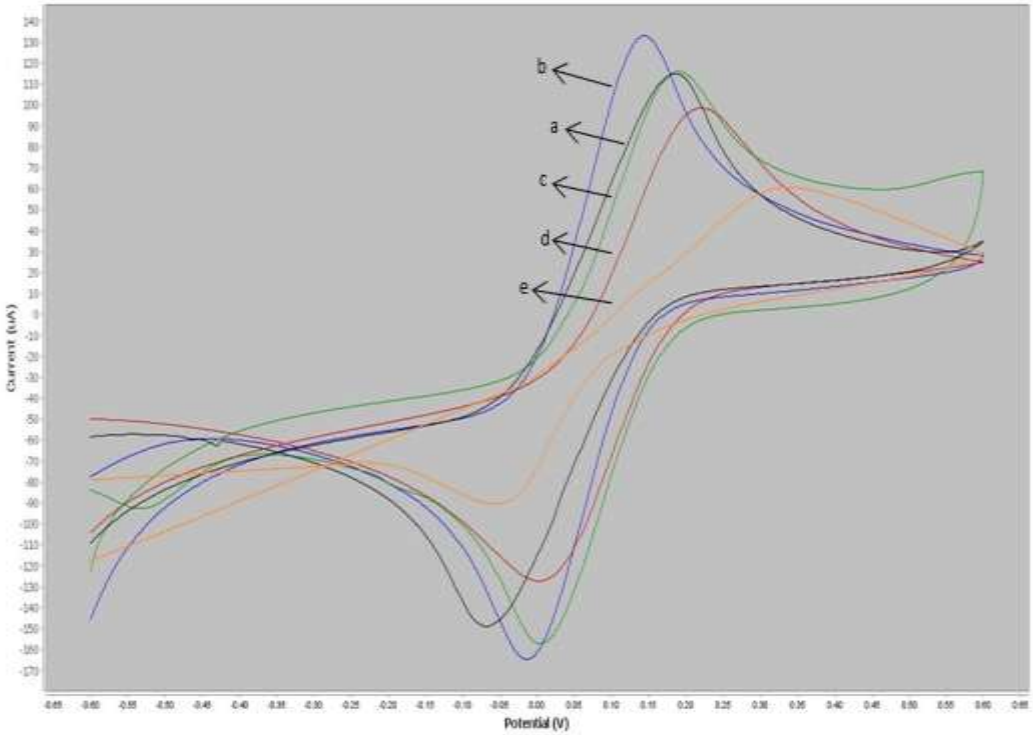

Figure 4: Cyclic voltammogram of electrochemical characterization of the modified electrode. In a solution of potassium ferricyanide 10 mM containing potassium chloride 0.1 M. Scan rate 0.05 V/s in the potential range -0.6 V to 0.6 V at stages: a) SPCE; b) SPCE/GNP; c) SPCE/GNP/MPA; d) SPCE/GNP/MPA/Ab; e) SPCE/GNP/MPA/Ab/Ag.

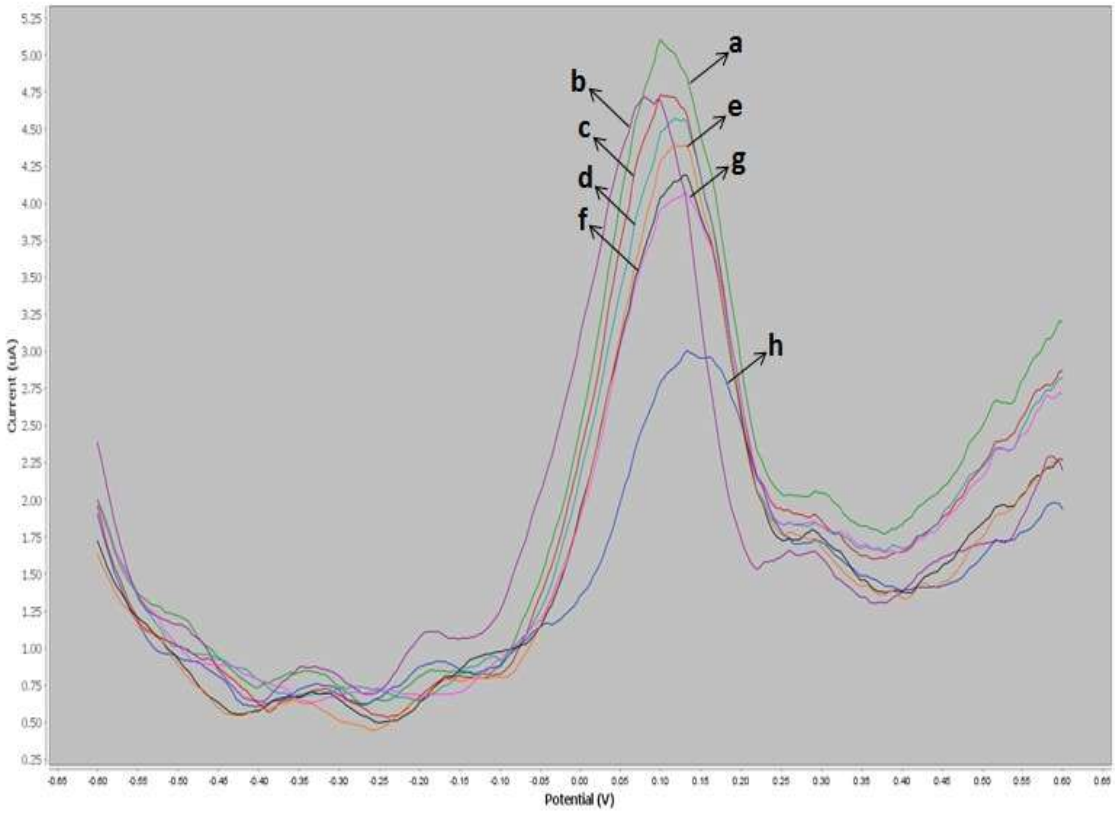

Figure 5: Voltammogram of differential pulse variation of HER2 concentration: (a) 0 ng/mL; b) 0.005 ng/mL; c) 0.01 ng/mL; d) 0.015 ng/mL; e) 0.02 ng/mL; f) 0.1 ng/mL; g) 1 ng/mL; h) 10 ng/mL. In a solution of potassium ferricyanide 10 mM containing potassium chloride 0.1 M. Scan rate 0.05 V/s in the potential range -0.6 V to 0.6 V.

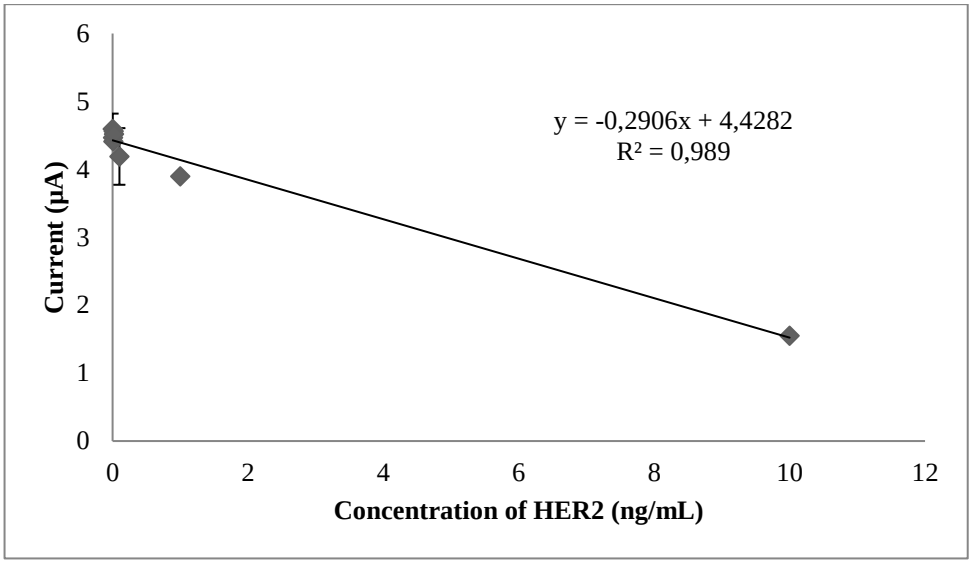

Figure 6: Calibration curve of immunosensor to HER2 antigen. HER2 concentrations: 0, 0.005, 0.01, 0.015, 0.02, 0.1, 1 and 10 ng/mL.

Conclusion

The results of this study show that anti-HER2 can be immobilized on the electrode surface by covalent bonds immobilization seen from the peak response of reduction-oxidation current of potassium ferricyanide on cyclic voltammogram on SPCE without and with gold modification via electrodeposition of chloroauric acid with an increase of 39.9 per cent and after immobilization anti-HER2 showed a decrease of 28.5 per cent. The sensitivity is 0.2906 for a concentration range of 0 to 10 ng/mL, LOD is 2.9 ng/mL, LOQ is 8.4 ng/mL, 89.7 percent of precision and 99.1 percent of accuracy.

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