Research Article

Evaluation of the role of N-Terminal non-glycosylated peptide (Fraction V) as multifunctional linker and study of its effect on the electrochemical behaviour of redox substrate by using Screen Printed Electrodes

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Abstract

Biological species detection by using screen printed electrodes (SPEs) is one of the important research areas for early-stage clinical diagnosis and environmental monitoring. Enzyme linked immunosorbent assay (ELISA) is widely used for determining bioanalytes such as antigen, antibody, DNA, glucose in the sample solutions. Moreover, it has potential uses in food and beverage industries for detecting food allergens, pathogens, etc. and chemical industries for detecting various metal ions at precise levels for quality control. In this study we evaluated the effect of antigen and antibody concentration on the electrochemical behaviour of 3, 3', 5, 5'tetramethylbenzidene (TMB) using commercial SPEs. We also investigated the role of N-Terminal non-glycosylated peptide (Fraction V) as a multifunctional linker between the electrode surface and the bioanalytes in the fabrication of the immunosensors. We also studied and compared the electrochemical response of TMB in three different commercial SPEs with the N-Terminal non-glycosylated peptide (Fraction V) as a crosslinker. This helps in the future development of Immunosensors for the diagnosis of many infectious and autoimmune diseases.

Keywords: Screen printed electrode; Immunosensors; Cross linker;3, 3', 5, 5'tetramethylbenzidene.

1. Introduction

In recent years fabrication of Immunosensors using SPEs gained much importance because of their low cost, portability, reusability, ease of use and are subjectable to automation [1,2]. They are important biological tools that replace the immunoassay techniques in different fields like, clinical diagnostics, environmental monitoring, food and biological industries. Selective interactions of immobilised molecules and unknown analyte of interest helps in the quantitative estimation and monitoring of the biomolecules like nucleic acids, protein molecules, drugs and metabolites, and the pathogens by an amperometric detector based on SPE. [3, 4]

Immunoassay can be performed in heterogeneous and homogeneous formats. In the heterogeneous format, the immune reactions happen on the surface of the substrate. Immobilisation of the antibody and antigen happens on a solid substrate forming the complex without further separation. Heterogeneous immunoassay has high surface area to volume ratio and excellent analytical sensitivity. But immobilising antibodies or antigen on the solid substrate is inextricable and sophisticated, for instance, hydrophilic protein molecules are difficult to absorb on the hydrophobic substrate without pre-treatment. This can be overcome by using hydrophilic

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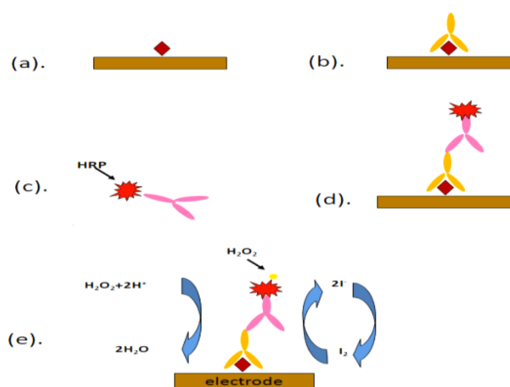


Figure 1. Block diagram (a). IgG immobilized on WE surface, (b). Primary anti –IgG interacts with immobilized IgG. (c). sec-IgG conjugated HRP enzyme (e) Electron transformation.

multifunctional linkers like N-Terminal non-glycosylated peptide (Fraction V). Moreover, N-Terminal non-glycosylated peptide (Fraction V) gained importance in the fabrication of immunosensors as it is capable of linking fatty acids, metabolites and drugs. N-Terminal non-glycosylated peptide (Fraction V) has many advantages that complement other cross-linking agents. Because of its low cost, solubility in aqueous media and its stability to increase the signal in the assays it is widely employed in the design of immunosensors. The negative charge on the molecule increases the ability to interact with different bioanalytes like water, salts, fatty acids, Vitamins and Hormones. Many biochemical reactions are not affected by N-Terminal non-glycosylated peptides (Fraction V). [3]

Depending on the assay format, the antigen or antibody is labelled and an enzyme activity measurement is performed as a final step of the assay. Different techniques like Fluorimetric, [5,6,7] luminometric and colorimetric [8, 9], electrochemistry [10,11,12] are used for the detection. One of the most important techniques is electrochemical detection of the immunoassay and it largely contributes to the future development of Immunosensors. In an immunoassay the signal responses generated by the substrates are electro reduced by the enzyme. Enzymes amplify the signal and catalyze the reactions. Two most commonly used enzymes are: horseradish peroxidase (HRP) and alkaline phosphatase (ALP) [13,14,15,16]. HRP-based sensors have higher background-current levels this is due to the interference caused by electro active substrates. Hydrogen peroxide (H2O2) is the critical and necessary oxidizing element in HRP catalysis sensor, which is prone to be electro reduced under a certain level of electrochemical potential window enhancing the background-current levels [14,17]. Thus, the electrochemical detection gained importance in recent years due to its precise sensitivity and due to its wide linear coverage [18]. This paper reports the effect of antigen and antibody concentration on the electrochemical behaviour of TMB using commercial SPEs. Impact of N-Terminal non-glycosylated peptide (Fraction V) as a multifunctional linker between the electrode surface and the bioanalytes in the fabrication of the immunosensor and comparison of the electrochemical behaviour of TMB on different commercial SPEs.

2. Principle:

SPEs consist of three electrodes, working electrode (WE) auxiliary electrode (AE) reference electrode (RE) fabricated in plane using electrically conducting material on an inert solid substrate. We fabricated the immunosensor by coating bioanalytes on the SPEs. At an applied sweep potential current flow between the WE and AE due to electron transformation in between the electrode surface and the sample solution. Current voltage characteristics plot indicates the rate of electrochemical reaction that takes place at the surface of WE. In this study we studied the electrochemical behaviour of TMB substrate by varying the concentrations of antigen and antibody coating on the SPEs. This helps to determine the effect of bioanalytes on the electrochemical response of the substrate and also to determine concentration of the specific analyte for the sensitive detection in ng ml-1 to fg ml-1 at a stable equilibrium condition. The Impact of N-Terminal non-glycosylated peptide (Fraction V) as a multifunctional linker on the electrochemical behaviour of TMB substrate was also investigated.

3. Experimental Work

3.1 Apparatus and Reagents:

Electrochemical characterization is carried out in galvanostat CHI-600E obtained from CH Instrument Inc (USA). Electrode surface morphology characterised by Field Emission Scanning Electron Microscopy (FESEM), Sigma HD, ZEISS, Germany. pH meter CL-51B, obtained from Utech Instrument Inc. India. Antigen: Goat IgG 1mg/ml (Purified from goat serum), blocking agent BSA in PBS-Tween, primary antibody: polyclonal rabbit antibody raised against goat IgG (3mg/ml), Secondary antibody: Rabbit antigoat IgG coupled to HRP (1000x), Substrate:3,3',5,5'tetramethylbenzidene (TMB)/H2O2 (20X), Coating buffer, Assay buffer, stop solution obtained from 3B Blackbio Biotech India Limited,India. N-Terminal non-glycosylated peptide (Fraction V) from Himedia laboratories.

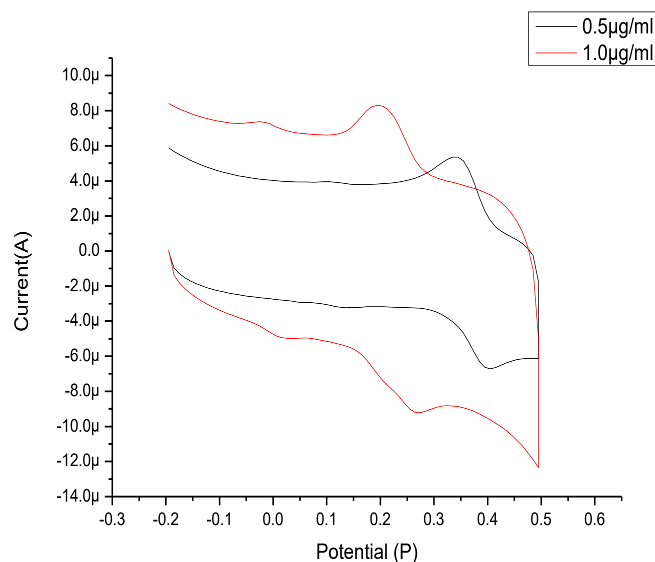


Figure 2. Electrochemical behaviour of TMB with 1µg/ml and 0.5µg/ml antigen concentration

Three commercial SPEs: C110 obtained from Dropsens Ltd, Spain; KS-540 obtained from Kanichi Research Ltd, UK; TE100 obtained from Zensor Research and Development, Taiwan.

3.2. Preparation of solutions:

1. 1X wash buffer (WB): To 10 ml of 20X wash buffer add 190ml of distilled water and store it at 4oC.
2. Blocking buffer (BB): To 1g of blocking reagent add 20ml of 1X wash buffer and warm it at low temperature.
3. Diluting buffer (DF): To 5ml of blocking buffer add 20ml of 1X wash buffer and mix well.
4. Substrate solution: To 20µl of TMB substrate add 980 µl of distilled water. Cover the tube with aluminium foil to avoid exposure to light.
5. Antigen: 1µg/ml and 0.5µg/ml of antigen was prepared by using the coating buffer.
6. N-Terminal non-glycosylated peptide (Fraction V): 1mg of N-Terminal non-glycosylated peptide (Fraction V) was weighed and dissolved in 1 ml of distilled water.
7. Secondary Antibody solution: 5µg/ml concentration of secondary antibody was prepared by using a diluting buffer.
8. Substrate solution: To 10µl of the 20X TMB substrate add 490µl of the distilled water.
9. Primary Antibody solution: 5µg/ml and 2.5mg/ml of primary antibody concentrations were prepared by using Diluent buffer.

3.3. Effect of Antigen concentration on the electrochemical behaviour of TMB substrate:

SPE carbon surface was coated with 25µl of 1µg/ml of IgG antigen solution which serves as a generic representative of any hypothetical protein or antigen (Figure1.) Then the electrode was incubated for 1hr to allow binding on to the SPE carbon surface, after which unbound antigen was washed from the electrode surface with a wash buffer for three times. Then 50µl of blocking buffer was added onto the electrode surface and incubated for 1hr at 37˚C to avoid non-specific binding of antibodies. A 25µl of primary antibody was added and incubated for 1hr at 37˚C. Then 25µl of secondary antibody conjugated with HRP was added and incubated for 1hr at 37˚C. Between all the above-mentioned steps a three-cycle washing step with 50µl wash buffer was adopted. Then 25µl of freshly prepared substrate solution was prepared and incubated for 5 min at 37˚C on the electrode surface. The above reaction was terminated by adding 50µl of stop solution. The CV response was recorded at applied sweep potential from -0.3mV to +0.6mV at 50mV/sec scan rate. The above-mentioned experiment was repeated with 0.5µg/ml of goat IgG antigen solution and CV response was recorded. All the other conditions were maintained similarly for both the electrode

In the CV response (Figure2.), it was observed that the electrochemical response pattern of both the concentrations is similar but there is a small shift in the potential of the low concentration peak towards the right. This is due to the non-specific binding of the antigen and the concentration of the intermediates generated in the reaction decreases with decrease in the antigen concentration. This supported the hypothesis of the relation between antigen immobilisation and the electrochemical response. Thus, the variation in the concentration of antigen leads to alteration in the electrochemical behaviour of TMB.

3.4. Effect of antibody concentration on the electrochemical behaviour of TMB substrate:

In this experiment carbon surface of two SPEs was coated with antigen concentration (1µg/ml).Then primary antibody of two different concentrations 5µg/ml and 2.5µg/ml were prepared

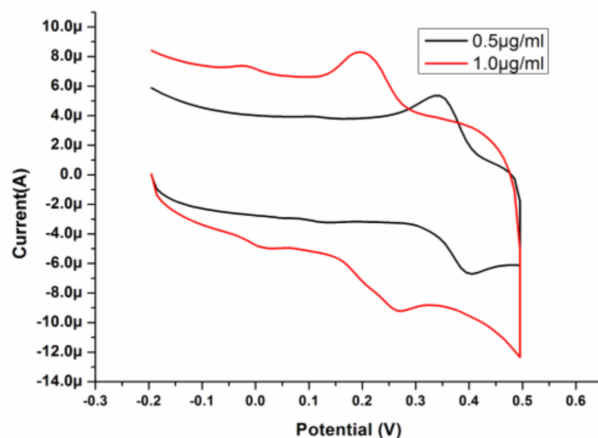


Figure 3. Electrochemical behaviour of TMB with 1µg/ml and 0.5µg/ml antigen concentration

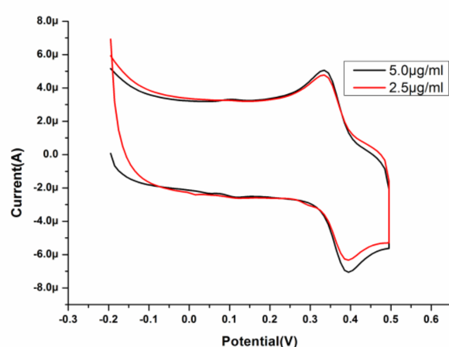


Figure 4. Electrochemical behaviour of TMB with 5µg/ml and 2.5µg/ml antibody concentration

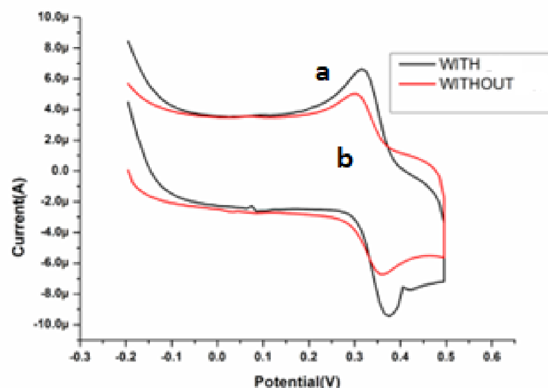


Figure 5. Effect of N-Terminal non-glycosylated peptide (Fraction V) on the electrochemical behaviour of TMB a. Electrochemical behaviour of TMB when Fraction V was used as linker(b) Electrochemical behaviour of TMB without Fraction V

and coated respectively. Then secondary antibody conjugated with the enzyme was coated on both the SPEs. Three cycle wash step was done between all the steps followed by incubation for 1hr at 37˚C.Then substrate solution was added and incubated for 5min at 37˚C and the reaction was terminated with 50µl stop solution. Electrochemical response of both the electrodes was recorded (Figure3.).

The electrochemical behaviour of the substrate followed the same pattern but there is minute decrease in the current magnitude of the peak with lower concentration of primary antibody due to the specificity and avidity of the antibody. This supported the hypothesis of the effect of primary antibody concentration on the electrochemical behaviour of TMB.

Impact of N-Terminal non-glycosylated peptide (Fraction V) on the electrochemical behaviour of TMB substrate.

The effect of N-Terminal non-glycosylated peptide (Fraction V) as a multi-functional linker on the electrochemical behaviour of TMB was studied in this experiment. We prepared 1mg/ml of N-Terminal non-glycosylated peptide (Fraction V) in distilled water and 50µl of solution was coated on the electrode surface and incubated overnight. When the above N-Terminal non-glycosylated peptide (Fraction V) treated electrode was used for the experiment 3.3 and CV response was recorded at applied sweep potential from -0.3mV to +0.6mV at 50mV/sec scan rate. (Figure4.)

The current magnitude of the N-Terminal non-glycosylated peptide (Fraction V) modified electrode was high when compared with the unmodified electrode (Figure5.). The pattern of the peak remained the same in both the graphs. This is due to the interaction of lysine residues of the N-Terminal non-glycosylated peptide (Fraction V) with the carbon increasing

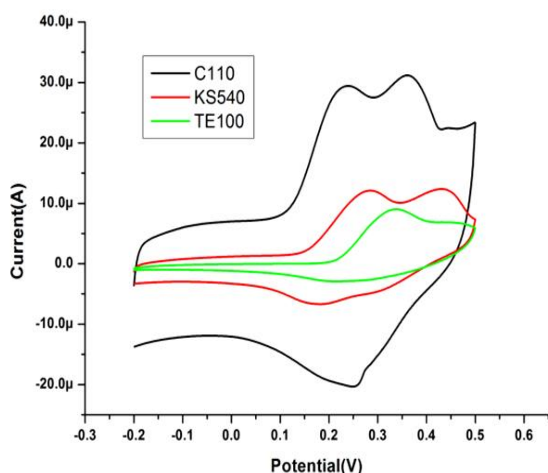


Figure 6. Electrochemical behaviour of TMB was studied on three different electrodes with N-Terminal non-glycosylated peptide (Fraction V) as cross linker.

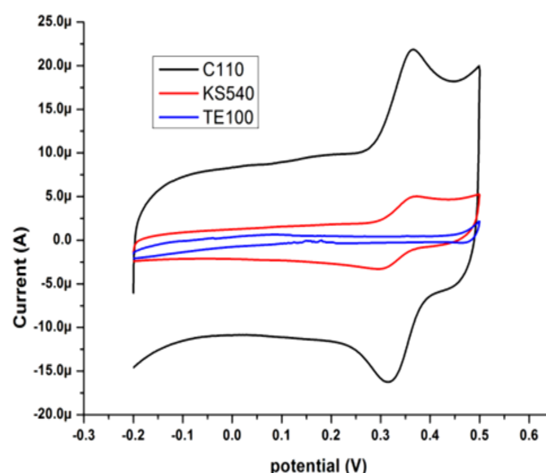


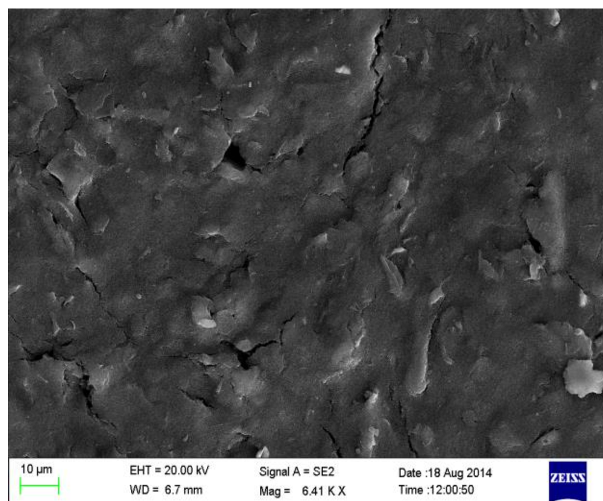
Figure 7. Electrochemical behaviour of TMB was studied on three different electrodes before treatment with N-Terminal non-glycosylated peptide (Fraction V).

the surface area for the antigen immobilisation. This supported the role of N-Terminal non-glycosylated peptide (Fraction V) as a multifunctional linker between carbon surface and antigen, as it increases the surface area of the carbon surface for the antigenic immobilisation.

3.6. Electrochemical behaviour of TMB substrate on three different SPEs:

In this study the experiment was done on three different SPEs electrodes C110, KS-540, TE100 obtained from Dropsens Ltd, Spain; Kanichi Research Ltd, UK; Zensor Research and Development Taiwan respectively. The experiment described in the 3.2 was done on three electrodes using antigen of concentration 5µg/ml.

The electrochemical response of three electrodes is different and this is due to the use of different composition of the graphite materials and procedures followed during fabrication of electrodes. In this study we studied the effect of N-Terminal non-glycosylated peptide (Fraction V) on the voltametric response. There is a difference in the pattern of the peaks before and after using crosslinker N-Terminal non-glycosylated peptide (Fraction V)Figure6 & Figure7. This change in the pattern of the peak was due to the cross linking of the bioanalytes with the N-Terminal non-glycosylated peptide (Fraction V).



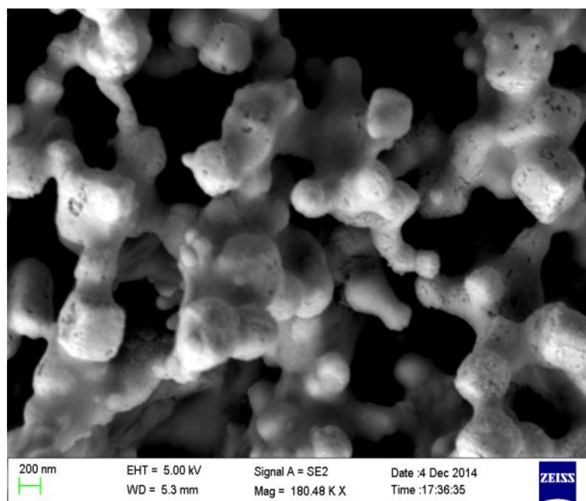
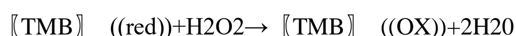


Figure 8. a) SEM image of the bare working electrode surface b) SEM image of the working electrode surface after treatment with bioanalytes and N-Terminal non-glycosylated peptide (Fraction V)

4. Results and discussion:

Electrochemical detection of the reduction current generated by the substrate helps to measure the activity of the enzyme. The enzymatic reduction product TMB (ox) is detected through its reduction at the surface of SPE.A working potential of -0.3mV to +0.6mV was selected for the measurement of HRP activity in order to obtain highly repeatable signals. At this potential there is low background current, no substrate oxidation and electrode stability are achieved. The electrochemical substrate TMB is oxidised when it reacts with hydrogen peroxide in presence of HRP enzyme generating reaction products. Oxidation of TMB in presence of H_2O_2 showed two electron electrochemical redox behaviour generating two peaks at 0.2mV and at 0.4mV due to the formation of intermediates in the reaction [19]. This is due to the two steps involved in the oxidation of the TMB which leads to the formation of cation and charge transfer complex. [20,21,22]



$$\text{〖TMB〗_((red))+H2O2→〖TMB〗_((OX))+2H20}$$

The capability of the immunosensor may be described in terms of sensitivity and precision. The sensitivity is the smallest amount which is measurable with a specified level of confidence [23]. The most important determinants of sensitivity and specificity in the immunosensor are the bioanalytes. The sensitivity of the immunosensor depends on the antigen and antibody concentration, their binding affinity and level of detection of the enzyme involved as a label. The affinity between the antigen and antibody limits the sensitivity of the immunosensor and therefore loss of sensitivity in immunosensor, may be ascribed to the decrease of the antigen and antibody binding affinity.

Critical factor in the fabrication of the immunosensors is the concentration and purity of the antigen. There is a shift in the peak potential of the graph 0.5µg/ml antigen concentration. (Figure2.) This is due to the non-specific adsorption of the antigen to the solid surface. The antibody specificity and avidity are important determinants in the preparation of immunosensors. Because of this property a very slight decrease in the current magnitude was observed (Figure3.). The role of N-Terminal non-glycosylated peptide (Fraction V) as a multifunctional linker was supported by the Figure5.There is increase in the current magnitude of the N-Terminal non-glycosylated peptide (Fraction V) modified electrode. This may be due to the increased surface area of attachment by N-Terminal non-glycosylated peptide (Fraction V) for the antigen immobilisation.

4.1 Electrode surface characterisation by microscopy:

Investigation of the morphological architecture of the scaffolds on the electrode surface after using N-Terminal non-glycosylated peptide (Fraction V) as a multifunctional linker and its association with antigen and antibody was carried out by FESEM.Figure8 (a) shows the SEM image of the bare working electrode surface. However, the surface was then covered with cloudy clusters due to the formation of N-Terminal non-glycosylated peptide (Fraction V), antigen and antibody complexesFigure8 (b).

5. Conclusion:

In the present study the bio-applications of N-Terminal non-glycosylated peptide (Fraction V) as a multifunctional linker in the fabrication of immunosensors were investigated by using different bioanalytes. The signal responses generated by the redox substrates were increased when N-Terminal non-glycosylated peptide (Fraction V) was used as a cross linker. The negative charge of the cross-linker may influence the binding affinity between the electrode surface and bioanalytes. Integration of SPEs and amperometry in the detection of bioanalytes by using cross linkers is possible with sensitive detection at a very low cost. The stability and charge of cross-linker may be responsible for the increased sensitivity of electrochemical response. Thus, N-Terminal non-glycosylated peptide (Fraction V) plays a key role of multifunctional linker and increases the affinity of the bioanalytes to the surface of the electrode for the fabrication of immunosensors. This helps in the future development of Immunosensors for specific biomarker measurement in the diagnosis of the disease.

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7. Conflict of Interest

The authors proclaim no conflict of interest.

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