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An Amperometric Genosensor for Detection of Virus Causing Marek's Disease in Poultry

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Abstract

The 5'-thiolated DNA probe of Meq oncogenic gene consisting of 24 deoxynucleotide, was reduced by Dithiothreitol (DTT) and immobilized covalently via thiolated bond onto gold electrode followed by hybridization with 1-4 ng/µl of Marek's Disease Virus (MDV) Single-Stranded (ss) DNA probe from tumor sample of chicken for 10 min at room temp. The Meqgenosensor could detect 1 ngss-DNA in tumor samples in min by Cyclic Voltammetry (CV), Differential Pulse Voltammetry (DPV) and Electrochemical Impedance (EI). The ssDNA modified Au electrode was characterized by Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). An amperometric genosensor was constructed for detection of Meq gene by connecting ssDNA electrode modified Au electrode (working electrode) with Ag/AgCl electrode (reference electrode) and Pt wire (as auxiliary electrode) through potentiostat. The hybridization between ssDNA probe and target ssDNA was detected by reduction in current generated by interaction of Methylene Blue (MB) with free G' of single stranded oligonucleotide. The ssDNA Au electrode showed optimum current within 10 min at pH8, 25^{0}C. This is the first report on construction of genosensor for the simple, fast and specific detection of MDV in chickens.

Keywords: Marek's disease virus; Genosensor; Poultry; Herpesvirus; Amperpmetric; Gold electrode

Introduction

Marek's disease is a highly contagious lymphoproliferative disease in chickens. Many types of birds may be affected by this disease but chickens are more prone to Marek's disease. Marek's Disease Virus (MDV) belongs to the genus Mardivirus, a member of Alphaherpesvirinae subfamily, Herpesviridae family [1]. Infection happens via inhalation of virus in respiratory tract, which further replicates in lungs and attacks adaptive immune system cells. It proliferates at maximum level after 10 days in feather follicle epitheilium cells genome. At this time infected chicken shed virus from skin and become an infectious agent for other chickens [2]. There are three serotypes, serotype-1 (Gallidherpesvirus 2), which is more virulent, followed by serotype-2 (Gallidherpesvirus 3) and serotype-3 (Meleagridherpesvirus 1). Meq gene is specific to serotype-1, which is responsible for oncogenesis in chickens. It causes paralysis in legs and wings while ocular lymphomatosiscauses graying of iris, nodular lesions on feather follicles [3]. In a study in India, it was confirmed with 173 chicken samples via PCR and in situ hybridization that the presence of highly virulent MDV strain in Indian poultry farms [4]. Marek's disease can be diagnosed by virus isolation [5], ELISA [6], PCR [7-13] nested PCR [14] real-time PCR [15-18] LAMP [19] and immunofluorescence [20]. But conventional diagnostic methods for MDV are time consuming and have various limitations. A study reported development of Loop mediated isothermal amplification for detection of MDV with 10 times more sensitivity than PCR [21]. Another study reported, a nested PCR was also developed to differentiate between oncogenic viruses to CVI988 strain which is attenuated form of virus [14]. HVT vaccine is widely used for the treatment of MDV in poultry [22]. So, genosensor could be a good diagnostic tool for MDV, as it is simple and very rapid. Meq gene is present within repeated region of MDV genome which encodes 339 amino acid trans-activator proteins (N-terminal basic region-leucinezipper, bZIP, C-terminal transactivation domain) responsible for cell transformation, also resemble with Fos/Jun family of oncoproteins [23]. It is responsible for the transformation of cells. In this study, a thiolated DNA probe specific for meq gene was selected, because of its importance in virulency and for its constant presence in oncogenic strain of MDV. It is easy to fabricate on gold wire and economically cheap. Methylene Blue (MB) was used as a redox indicator for electrochemical genosensor [24,25]. FTIR and SEM were used for characterization of modifications on biosensor at different stages to confirm the presence of functional groups specific for that modification. This sensor is made to detect even a small amount of DNA to confirm the presence of virus.

Materials and methods

Chemicals and sample

The 5'-thiolated ssDNA probe (5'-ATACCACGCCAACGAAAAGAATGT-3') was synthesized on commercial basis using Integrated DNA Technologies (IDT), Faridabad, India. Electrodes (gold: working electrode, Ag/AgCl as reference and Pt wire as counter electrode). All the chemicals used were of AR grade. Tumor samples with suspected Marek's disease chickens were obtained from Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar. India the double Distilled Water (DW) was used through the study.

Isolation of DNA from tissue sample

Tumor tissue containing MDV was homogenized in 1 ml of extraction buffer (50 mM tris-HCl pH8, 25 mM EDTA, 400 Mm NaCl) for 5 min. Then 10 µl of proteinase K was added followed by addition of 300 µl of 10% SDS and then mixed well and incubated at 65^{0}C in DW bath for 3 hr. After incubation, equal volume of mixture of Phenol/Chloroform/Isoamyl (PCI) in 25:24:1 ratio was added and mixed well, followed by centrifugation at 13,000 rpm for 10 min at 4^{0}C. Aqueous phase was created, which was transferred into a new micro-centrifuge tube. After this equal volume of chloroform: isoamyl

alcohol in 24:1 ratio was added and mixed and then centrifuged at 13,000 rpm for 10 min at 4^0C. The aqueous phase was transferred in to a new micro-centrifuge tube and 1:10 volume of 3M sodium acetate was added followed by addition of 0.6 volume of isopropyl alcohol and then mixed gently. Thread like structure of DNA was seen in solution at this stage and then centrifuged again at 13,000 rpm for 10 min at 4^0C. Supernatant was discarded and the pellet was washed with 1 ml of 70% ethanol followed by centrifugation at 13,000 rpm for 10 min at 4^0C. Then ethanol was discarded and pellet was dried. The pellet was re-suspended in 100 µl of nuclease free water and incubated at 65^0C in water bath for 10 min to dissolve the pellet.

The Genomic DNA (G-DNA) of MDV, isolated from sample, was denatured at 95^0C for 5 min to make Single Stranded DNA (ssDNA) to get 1 ng/µl, 2 ng/µl, 3 ng/µl and 4 ng/µl for hybridization with the immobilized probe on gold wire. The G-DNA of MDV was also tested by PCR.

Reduction of 5'-thiolated probe

The 5'-thiolated probes were reduced by Di-Thiothreitol (DTT) reduction method. To reduce, 100 mM DTT solution in sodium phosphate buffer (pH8.3-pH8.5) was prepared. DTT powder (77.13 mg) was added to 5 ml of 100 mM solution. Then, 400 µl of 100 mM added directly to lyophilized thiolated probe and left at room temperature for 1 hrs. to reduce the thiol groups and vortexed. After this, 50 µl of 3 M sodium acetate pH5.2 added and vortexed. Now 1.5 ml of absolute ethanol was added, vortexed and stored at -80^0C for 20 min, followed by centrifugation at 12,000 rpm for 10 min. decanted ethanol and pellet was air dried. Pellet was dissolved by adding 200 µl of sterile nuclease free water or buffer. At last, concentration by an absorbance at 260 nm was taken in nano drop. The concentration of probe was 1288.2 ng/µl.

Fabrication of genosensor

To prepare working electrode, an Au wire (2 mm × 20 mm) (diameter × height) was surface activated by treating it with H2SO4/H2O2 (1:1, v/v) for 5-7 min and then washed with autoclaved double distilled water, followed by absolute ethanol and then with distilled water and finally dried at room temperature. The ssDNA-SH was immobilized onto polycrystalline Au wire by dipping it into 100 µl of 5'-thiolated DNA probes (10 µM) for 24 hours at 25^0C. Unbound DNA probe was removed by washing modified Au wire with Phosphate Buffer Saline (PBS) pH7 and dried at room temperature for 2-3 hours. The ssG-DNA 1-4 ng/100 µl from chicken tissue sample was hybridized at 25^0C for 10 min with ssDNA-SH/Au. After hybridization process, electrode was washed with TE buffer (Tris-HCl and EDTA) for 3-5 times, pH8 to remove unhybridized meqssG-DNA and again washed with PBS, pH7 before doing electrochemical study. The immobilization of probe onto gold wire and hybridization with ssG-DNA of virus was detected by CV, DPV and EI using FRA2 µAUTOLAB (type of device). This was repeated three times and average values were taken to plot graph between concentration of ssG-DNA and oxidation peak current (Ip). The scheme of fabrication of genosensor onto Au wire is shown in Figure 1.

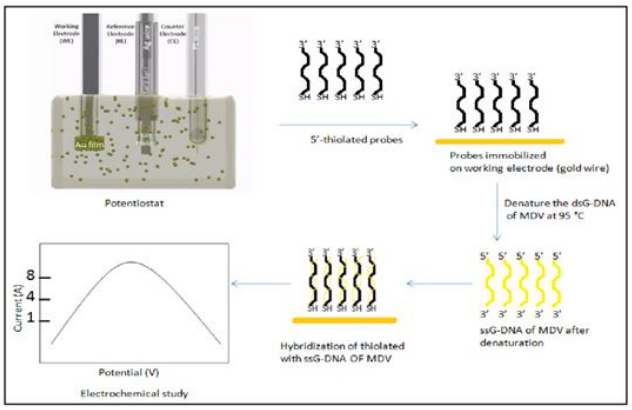

Figure 1: Scheme of immobilization of 5'-thiolated meq ssDNA probe on Au electrode and hybridization with ssG-DNA of MDV from sample.

Results and Discussion

CV studies

Figure 2 and Figure 3 shows the CV of ssDNA-SH/Au and dsDNA-SH/Au. The CV current of bare Au (20 µA) was lower than the current of ssDNA-SH/Au (33.8 µA), due to higher rate of electron exchange between methylene blue and gold wire. Whereas, electron exchange was decreased because of strong association of Methylene Blue (MB) with unpaired Guanine of ssDNA-SH/Au, so, total output of current decreases. The oxidation peak current of dsDNA-SH/Au was 48.38 µA, 50.43 µA, 80.72 µA and 135.3 µA after hybridization with 1, 2, 3 and 4 ng/µl of ssDNA of G-DNA of MDV respectively. Oxidation current increased as ssG-DNA increased because of the presence of extra unhybridized guanine of G-DNA which interacted with MB and gave increased output of current.

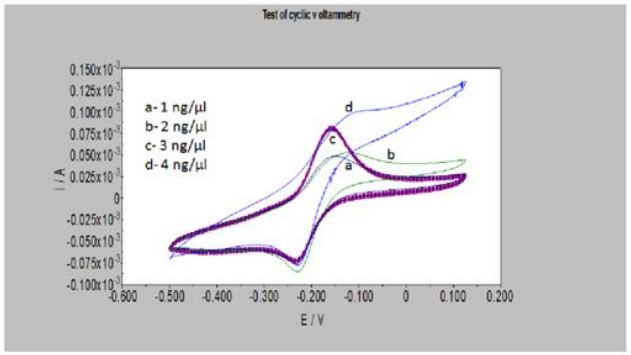

Figure 2: Cyclic voltammograms of ssDNA-SH/Au hybridization with 1, 2, 3 and 4 ng/µl of ssG-DNA using 1mM MB in 100 ml PBS, pH 7.

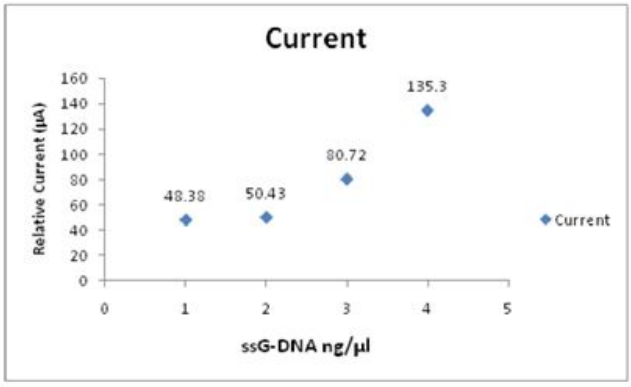

Figure 3: Graph showing relationship between relative current (Ip) and ssG-DNA of samples.

DPV studies

The DPV current of ssDNA-SH/Au (92.8 µA) was higher than bare (33.5 µA) gold wire. The change of current is similar as like in CV studies. The DPV current of dsDNA-SH/Au increased with increase in concentration of ssG-DNA of MDV, DPV in sample 1, 2, 3 and 4 ng/µl was 105 µA, 122 µA, 153 µA and 188 µA (Figure 4).

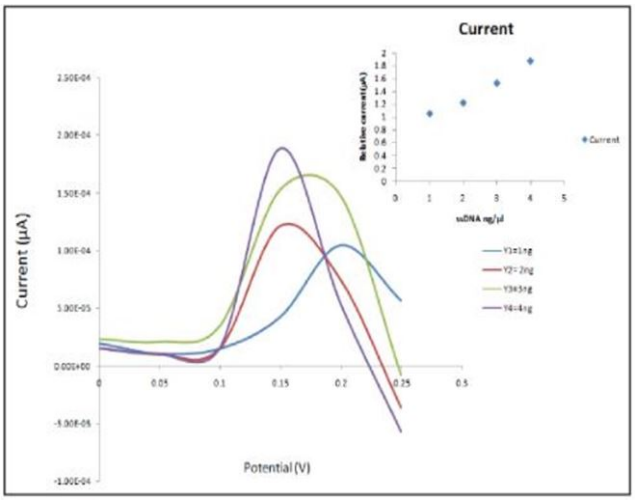

Figure 4: Differential pulse voltammetry of ssDNA-SH/Au hybridization with 1, 2, 3 and 4 ng/µl ssG-DNA using 1 mM MB in 100 ml PBS, pH7. The inset shows increase of relative peak current (Ip) value of ssG-DNA of MDV.

EI studiess

The Nyquist plot of ssDNA-SH/Au and hybridization of different concentrations of ssG-DNA is shown in Figure 5. The diameter of semi-circle gives value of Charge Transfer Resistance (Rct) at the electrode interface, whereas linear region defines value about diffusion of electrons. The Rct value of bare (3.5 kΩ) electrode is lower than ssDNA-SH/Au (8.2 kΩ) because of probe bounding to electrode, which prevented the (Fe (CN) 6) 3-/4- ions from reaching to surface of electrode. The Rct value of dsDNA-SH/Au with 1 ng/µl, 2 ng/µl, 3 ng/µl and 4 ng/µldilutions were 8.6 kΩ, 9.78 kΩ, 9.82 kΩ and 10.7 kΩ the extra deoxyribose backbone of unhybridized DNA increased the repulsion with (Fe (CN) 6) 3-/4- ions resulting in increased Rct value of the dsDNA-SH/Au.

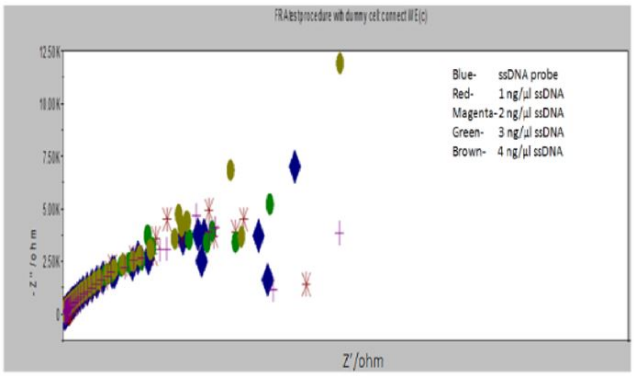

Figure 5: Nyquist plot for ssDNA-SH/Au (blue) and hybridization with 1 ng/µl (red), 2 ng/µl (magenta), 3 ng/µl (green) and 4 ng/µl (brown) of ssG-DNA of MDV using 5 mM PBS, pH 7.

Characterization of genosensor (FTIR and SEM)

The hybridization of ssG-DNA with immobilized probes was characterized by FTIR spectra on Au electrode. The immobilized probe showed peaks at and hybridization peaks at 592 cm-1, 1115 cm-1, 1019 cm-1 and 799 cm-1 corresponding to thiamine (C2=O stretching), adenine (C7=N vibration), cytosine (in-plane vibration of cytosine) and guanine (C=O stretching), respectively shows the presence of all four nucleotides [26] Figure 6 showing FTIR transmission spectra of dsDNA (a) and ssDNA (b) at frequency 400-4,000 cm-1.

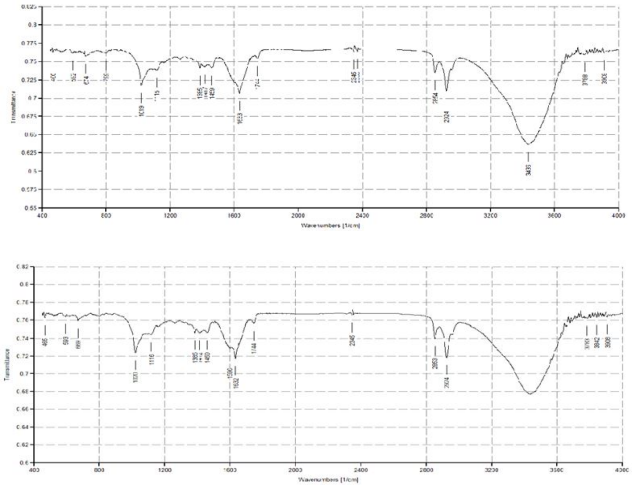

Figure 6: FTIR transmission spectra of (a) ds-DNA and (b) ssDNA at frequency 400-4,000 cm-1.

The SEM images showed roughness of bare gold, which was further increased as the immobilization of probe has done in ssDNA-SH/Au and this roughness increased again with hybridization of sample. This change in roughness on gold wire confirmed the immobilization of ssDNA-SH/Au probe and hybridization in case of dsDNA-SH/Au. Figure 7 showing the increase in roughness from bare electrode (7A), ssDNA-SH/Au (7B) and dsDNA-SH/Au (7C) gold electrodes respectively.

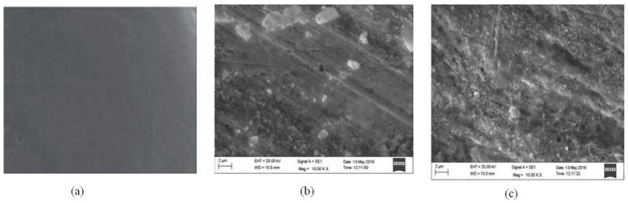

Figure 7: SEM images of bare (7A), ssDNA-SH/Au (7B) and dsDNA-SH/Au (7C) gold electrodes.

Application of genosensor

The genosensor was applied to detect maq gene in 8 muscle tissue samples of poultry suspected with Marek Disease Virus (MDV) in which 2 samples were found negative and 6 samples were found positive. The change in IPof DPV with different concentration of genomic DNA of virus confirms our result. These results were also confirmed by the PCR (Figure 8).

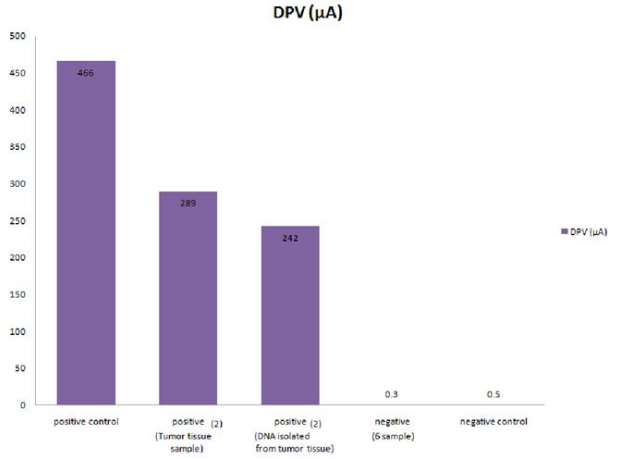

Figure 8: Relative peak current of DPV in µA of different samples.

Conclusion

An amperometric genosensor was constructed for the detection of Marek Disease Virus (MDV). The sensor could detect as low as 1 ng/µl ssG-DNA of MDV in sample within 30 min to diagnose the disease. This is very specific, because it is based on DNA probe for Meq gene, which is present only in specific oncogenic virulent strain. It is also better than other detection methods, which require expensive instrument and are not easy to handle. To the best of our knowledge, this is very first genosensor for the detection of Marek's disease in poultry industry.

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