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IMMOBILIZATION OF HEPATITIS B ANTIBODY ONTO THE REDUCED GRAPHENE OXIDE

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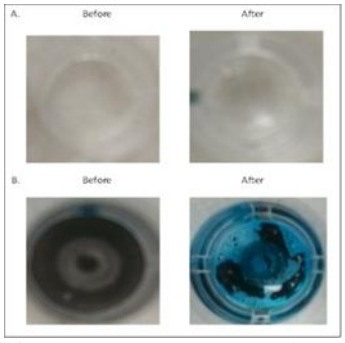
Graphical abstract



(A) Without the presence of rGO-nafion (B) Presence of rGO-nafion. Both undergo the complete immobilization process.

Abstract

Hepatitis B (Hep B) is a viral infection caused by hepatitis B virus (HBV) that infects the human liver. HBV is blood-borne and transfusion-transmitted human pathogen that has a large impact on blood safety and public health worldwide. Approximately 5% of the Malaysian population is infected by HBV. The treatment to this disease is possible if early detection is done before irreversible liver damage occurs. The presence of hepatitis B virus surface antigen (HBsAg) is an evidence of the presence of HBV infections. Presently, the conventional methods used for detection show weaknesses in terms of time and efficiency. In this research, the functionality of reduced graphene oxide (rGO) as an immuno biosensor for the detection of HBsAg has been studied. The graphene oxide was prepared via Modified Hummer's Method due to its ability to produce graphene in large quantity. The fabrication of immunosensor was conducted by immobilizing antibody onto the graphene. nafion-graphene nanosheet was grown and later treated with thionine solution. Following that, a solution of primary antibodies against Hep B surface antigen (anti-HBsAg IgG) was incubated on the biosensor. After the immobilization, the antigen was dropped onto the biosensor, followed by incubation of secondary antibodies conjugated to Horseradish Peroxidase (HRP). Upon addition of Tetramethylbenzidine (TMB), the colour of the solution changes to blue. Thus, proving the functionality of rGO as an immunobiosensor for the detection of HBsAg.

Keywords: Biosensor, graphene, hepatitis B surface antigen

Abstrak

Hepatitis B (Hep B) adalah jangkitan virus yang disebabkan oleh virus hepatitis B (HBV) yang menjangkiti hati manusia. HBV berjangkit melalui transfusi darah dan ini mempunyai kesan yang besar pada keselamatan darah dan kesihatan awam di seluruh dunia. Kira-kira 5% daripada penduduk Malaysia dijangkiti HBV dan rawatan terbaik adalah untuk pengesanan awal sebelum kerosakan hati berlaku. Kewujudan antigen permukaan Hepatitis B (HBsAg) adalah bukti kehadiran jangkitan HBV. Pada masa ini, kaedah konvensional yang digunakan untuk pengesanan menunjukkan kelemahan dari segi masa dan kecekapan. Dalam kajian ini, kami berhasrat untuk mengkaji tentang kemungkinan 'reduced graphene okside' (rGO) sebagai bahan asas untuk biosensor imun untuk mengesan HBsAg. rGO disediakan melalui Kaedah Modified Hummer kerana kemampuannya untuk menghasilkan graphene dalam kuantiti yang besar. Fabrikasi immunosensor telah dijalankan dengan memperkenalkan antibodi kepada graphene. nafion-graphene serpihan dirawat dengan larutan Thionine. Berikutan itu, satu larutan antibodi utama Hep B antigen (anti-HBsAg IgG) telah dieram pada biosensor terdahulu. Selepas itu, antigen yang digugurkan ke biosensor, diikuti oleh pengeraman antibodi sekunder konjugasi dengan 'Horseradish Peroxidase' (HRP). Berikutan dengan penambahan Tetramethylbenzidine (TMB), warna larutan akan berubah kepada biru. Sebagai kesimpulan, rGO boleh digunakan sebagai bahan asas untuk biosensor.

Kata kunci: *Kata kunci*: Biosensor, graphene, hepatitis B surface antigen

1.0 INTRODUCTION

Hep B is a disease caused by HBV that attacks the liver. A HBV infection can cause both acute and chronic diseases,[1]which is a major health problem occurring in all parts of the world.[2] The World Health Organisation,[3] mentioned that approximately 780 000 people die each year due to the acute or chronic consequences of Hep B. In Malaysia, 5% of its population is affected by Hep B.[4] Hence, the best treatment for Hep B is to eliminate the virus before the occurrence of irreversible liver damage. For this to be successful, accurate detection of HBV infections in the early stages is crucial. The conventional methods of detecting HBV such as ELISA and PCR is time consuming,[5] hence, it is essential to identify low cost, rapid, high selectivity and sensitivity bio-diagnosis markers present at ultra-low level during early stages of infections. Therefore, the research on graphene based biosensor for the purpose of early detection of HBV is crucial in terms of cost and effectiveness.

Graphene is much favored for the usage as a biosensor due to its unique physicochemical properties such as high surface area and extraordinary electron transfer ability,[6] which are useful for the purpose of a biosensor. Previous studies have proven that further reduction of graphene oxide improved the properties exhibited by graphene.[7-9] The presence of a large number of oxygen-containing functional groups and structural defects[10] enhanced rGO's chemical activity compared to graphene.

AIM: To study on the functionality of rGO as as immuno-biosensor for the detection of HBsAg.

2.0 EXPERIMENTAL

2.1 Material

Nafion 117 solution and thionine acetate salt were obtained from Sigma Aldrich. The hepatitis B virus surface antigen (HBsAg) was purchased from MP Biomedicals Asia Pacific Pte. Ltd. with the product name Purified human HBsAg subtype ad (Article No. COMM-9002). The primary antibody Hepatitis B surface antigen antibody (HRP) (Cat. No. GTX19990), secondary antibody, Hepatitis B surface antigen antibody [HB6] (HRP) (Cat. No. GTX40106) and tetramethylbenzidine (TMB) were purchased from Gene Tex.

2.2 Experimental Procedures

2.2.1 Synthesis of Reduced Graphene Oxide (rGO)

The rGO was prepared by using Modified Hummer's Method.[11-13] The prepared rGO was kept in a vacuum desiccator until used. Characterization of rGO was conducted by means of Ultraviolet- Visible (UV-Vis) Spectrometer and Fourier Transform Infrared Spectroscopy (FTIR). UV-Vis was conducted to observe the reaction process of the production of rGO, [14] whereas FTIR characterization was conducted to determine the functional groups present in rGO.[15]

2.2.2 Functionalization of Reduced Graphene Oxide (rGO)

The rGO was used to grow the rGO-nafion nanosheet by ultrasonication in 0.25% nafion-water for 30-50 minutes to obtain a homogenous, well distributed suspension of rGO-nafion composite. The prepared rGO-nafion solutions were stored at room temperature until time of use.

2.2.3 Immobilization of Hepatitis B Surface Antigen (HBsAg)

100 uL of rGO-nafion was added onto a treated surface and allowed to dry to fabricate the biosensor. Next, thionine solution (3mM) was added onto the surface and allowed to soak for 10 minutes. The treated biosensor surface is then incubated with primary antibody (100 µL) at 37°C for half an hour. The antibody was then removed and the sample was washed (5X) with 1X PBS (250 µL/well), approximately1 minute per wash. The non-specific binding of the primary antibody is the blocked with 1% BSA/PBS (200 µL/well) and incubated further for an hour at 37°C. The fabricated biosensor was sealed and frozen at -70°C until use.

Figure 1 shows the methodology developed for the detection of HBsAg using the rGO biosensor. The antigen was introduced onto the sensor and incubated for half an hour at room temperature and followed by a similar washing process (5X) with1X PBS. The sensor was then incubated with the secondary antibody (conjugated with HRP) for 30 minutes at room

temperature and followed by washing (5X) with 1X PBS. Next, TMB (100 µL/well) was incubated for 15 minutes. TMB is a chromogenic substance used in the staining procedures of immunohistochemistry as well as a visualizing reagent for enzyme-linked immunosorbent assays (ELISA) and reading was taken at 450 nm.[16] The immobilization of HBsAg was replicated three times.

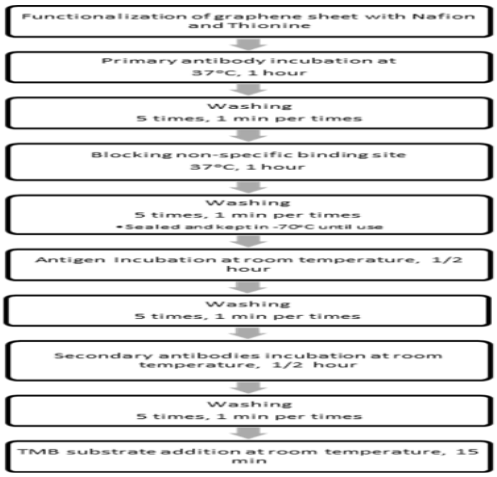


Figure 1 Methodology for detection of HBsAG using the RGO biosensor

3.0 RESULTS AND DISCUSSION

3.1 Characterization

The yellowish brown liquid (Figure 2(A)) shows the dispersion of graphene oxide (GO) in water before reduction. This viscous solution contained graphite oxide and exfoliated sheets as well as non-oxidized graphitic particles and residues of the oxidizing agents from the reaction mixture.[15] The black solution in Figure 2(B) represents the rGO. The rGO formed was then filtered and washed profusely with water and finally with acetone. The rGO precipitation (powder) obtained was later be functionalized with 0.5% nafion as seen in Figure 2(C).

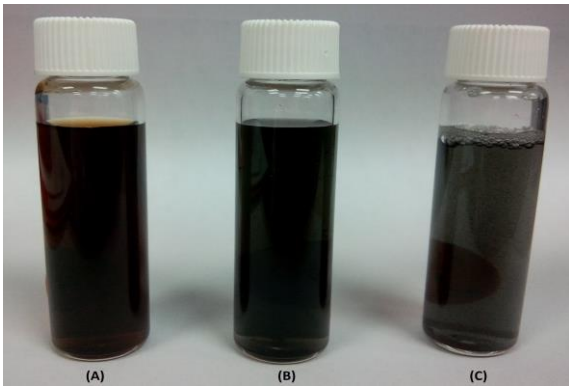


Figure 2 Dispersion of (A) graphene oxide (B) reduced graphene oxide) (C) reduced graphene oxide – 0.5% nafion

The characterization of the produced rGO was conducted via UV-Vis spectroscopy and FTIR. In Figure 3, the UV-Vis absorption spectra of rGO before and after undergoing reduction are shown. The absorption peak shifted from 230 nm to 265 nm[14,17]. The shift in peak is due to the electronic conjugation within the reduced graphene sheets upon the reduction of graphene oxide in the presence of hydrazine.[14,17]

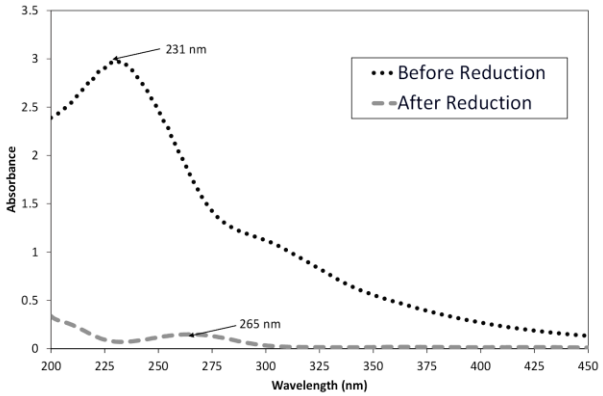


Figure 3 Graph of absorbance vs. wavelength obtained from UV-Vis Spectroscopy shows the peak shift from 231nm to 265nm

Figure 4 shows the FTIR spectra of graphite and rGO. Graphite spectra produced insignificant peaks while rGO spectra showed the presence of different functional groups Among them are hydroxyl (O-H) group at 3236.03 cm^{-1}, alkane (C-H) group at 2959.82 cm^{-1} and 2917.50 cm^{-1}, carboxyl (R-C(O)OH) group at 1698.58 cm^{-1}. Carboxyl group is still present in which it is suggested that the aqueous solution of rGO is still charged even after reduction. [14]

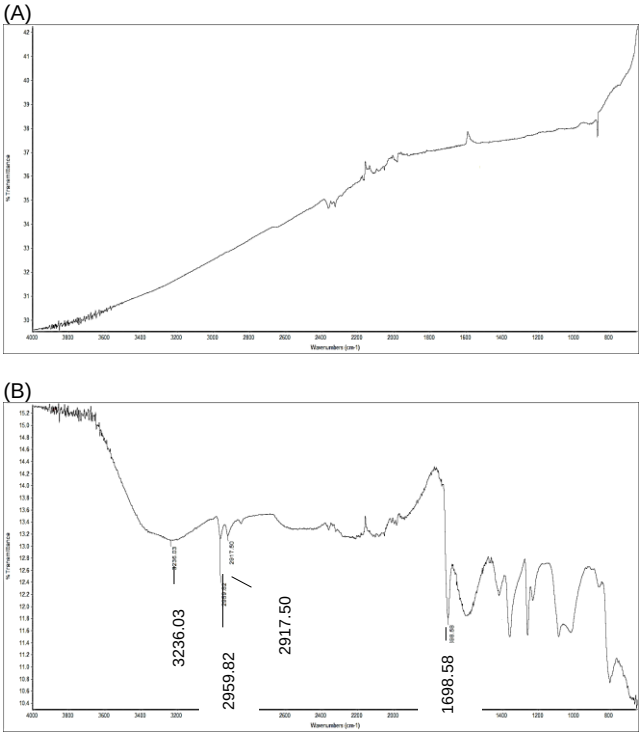


Figure 4 FTIR spectra of (A) graphite and (B) reduced graphene oxide (rGO)

3.2 Immobilization of Hepatitis B Surface Antigen (HBsAg)

The binding of HBsAg with antibody was confirmed with the introduction of TMB substrate at room temperature for 15 minutes. As shown in Figure 5, TMB substrates detected HRP activity, thus yielding a blue color solution.

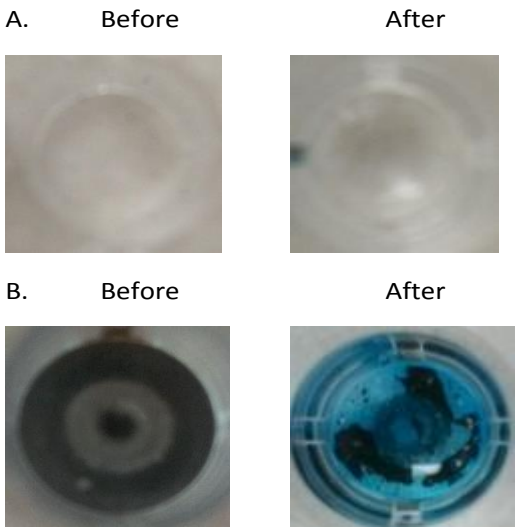


Figure 5 (A) Without the presence of rGO-nafion (B) Presence of rGO-nafion. Both undergo the complete immobilization process

To summarize, the developed immobilization process required approximately 1 hour and 25 minutes in order for the Hep B antigen to be detected. From the results obtained it shows that rGO could be a potential matrix as an immunosensor. However, further study is advised to further confirm the functionality of rGO as an immune biosensor. Electrochemical characterization of the functionalized rGO as an immunosensor can be developed for better understanding on the biocompatibility of rGO.

4.0 CONCLUSION

The development of an rGO based immunoassay for the detection of HBsAg is feasible as shown in the results obtained. The colour-change obtained from visual observation has proven the functionality of rGO as an immune-biosensor. This is probably due to the unique characteristics, [11] of having high surface area and also the presence of active sites which are used for the binding of antibody which will attach the respective antigen.

From the results obtained, without the presence of rGO, anti-HBsAg IgG is unable to bind to the surface as there is no rGO present. Hence, with the presence of rGO functionalized with the anti-HBsAg IgG is able to attach the HBsAg. TMB is a colourless substance which only changes color in the presence of HRP contained in the secondary antibody.

However, it is recommended that further study is required to have better understanding on the functionalized rGO as an immunosensor.

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