Improvement on Reusability, Storage Stability and Thermal Stability of Magnetic Graphene Oxide- Immobilized Cholesterol Oxidase

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Improvement on Reusability, Storage Stability and Thermal Stability of Magnetic Graphene Oxide-Immobilized Cholesterol Oxidase

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Abstract: Biosensor using the enzyme cholesterol oxidase (ChOx) was one of the cholesterol detection methods commonly developed because of its good specificity. Immobilization would increase the stability of the enzyme, hence magnetic graphene oxide was chosen as support material because of its high surface area, good thermal conductivity, high number of functional groups, and paramagnetic properties. This work aimed to study the effects of using magnetic graphene oxide as support for the immobilization of cholesterol oxidase. Magnetic graphene oxide was synthesized, and cholesterol oxidase was crosslinked to the material. The immobilized enzyme was confirmed by the appearance of the P-O-C group at a wavenumber of 920 cm^{-1} and a C=O group at a wavenumber of 1724 cm^{-1}, accompanied by the surface structure of the material becoming much rougher. The result revealed that after ten cycles, immobilized ChOx maintained 7.10% of its initial activity. The activity retained after being kept at 4ºC for 10 days for immobilized and free ChOx were 97.68% and 36.25% of its initial activity, respectively. The immobilized enzyme also showed better thermal stability, with immobilized and free ChOx respectively retaining 88.35% and 58.05% of its initial activity after incubated at 70ºC.

Keywords: biosensor, cholesterol, cholesterol oxidase, immobilized enzyme, magnetic graphene oxide

1. Introduction

Although cholesterol is needed in the body, the negative effects of high cholesterol levels on the human body have been well documented. High cholesterol levels cause clotting on the artery walls, thereby narrowing the blood flow path and causing an increased risk of heart disease[1)]. Data from Basic Health Research stated that the incidence of heart disease in Indonesia was increasing year by year, and in 2018 it reached 2,784,064 sufferers. Therefore, to help people maintain cholesterol levels in the blood, cholesterol detection methods are important.

A cholesterol detection method has been developed using a biosensor. Enzyme-based biosensors have various advantages, including high sensitivity and specificity, portability, low cost, and the possibility for point-of-care testing[2)]. In the manufacture of cholesterol biosensors, the enzyme cholesterol oxidase (ChOx) is most commonly used. Cholesterol oxidase enzyme can specifically oxidize cholesterol to 4-cholesten-3-one and hydrogen peroxide. The amount of hydrogen peroxide produced is then measured so that the cholesterol levels can be determined[3)].

Numerous researchers had been modified bio-resource with the other materials to provide better properties[4)]. Modification of enzyme immobilization is an interesting topic for researchers because it is an effective way to increase the stability and reusability of enzymes[5)]. Enzyme immobilization is a crucial modification and also can be stated as the confinement of an enzyme molecule to physical or chemical support or both, so that the enzyme activity can be fully or largely maintained. It is one of the key factors affecting the stability, sensitivity, and selectivity of biosensors[6)]. The immobilization of this enzyme will reduce the costs that must be incurred for the enzyme because the enzyme can be reused many times[7)].

The type of support material is a factor that is considered to affect the effectiveness of the enzyme immobilization process[8)]. Several support materials that

have been used to immobilize enzymes in the development of biosensors are cellulose acetate, sol-gel, polyvinyl chloride, perlite, etc., including chitosan and silicon dioxide that was studied as ChOx support previously [8),9)]. Out of the support materials that have been used to immobilize enzymes, graphene oxide is considered to be a promising one for biosensor applications[10)].

The graphene oxide material has ideal properties for enzyme immobilization such as high surface area, chemical stability, good thermal conductivity, and a large number of functional groups that facilitate interactions with many biomolecules. However, enzymes immobilized in these materials are difficult to recover due to their low hydrophobicity. Therefore, the material needs to be modified and combined with magnetite to facilitate enzyme recovery due to its paramagnetic properties.

Magnetic graphene oxide material is well-established as support for glucose oxidase enzymes for glucose biosensor applications. The composite between graphene oxide and magnetite in addition to expanding the surface area, also facilitates the prevention of enzyme leaching on the electrode surface because it can be manipulated using an external magnetic field. This support material was also found to increase the contact between the active site of the enzyme and the electrode surface, thus facilitating the biosensing process[11)]. Magnetic graphene oxide material has been used to immobilize several other enzymes such as lipase, horseradish peroxidase, and glucose oxidase, but its use for cholesterol oxidase has not been recorded.

Based on the discussion above, this research was conducted to further develop variations of the type of cholesterol oxidase enzyme support for biosensors. The purpose of this research is to immobilize the enzyme on magnetic graphene oxide material and use it to oxidize cholesterol. In this research, a study of reusability, storage stability and thermal stability have been carried out.

2. Materials and Apparatus

The following chemicals were obtained from commercial sources: cholesterol oxidase 20 U/mg (Sigma-Aldrich), cholesterol (Sigma-Aldrich), aquadest, graphene oxide, ammonia, ethanol (Merck), glutaraldehyde (Merck), triton x-100 (Sigma-Aldrich), phenol, 4-aminoantipyrine, horseradish peroxidase 150 U/mg (Sigma-Aldrich), phosphate-buffered saline 0.01 M pH 7.4 (Sigma-Aldrich), and 3-aminopropyl triethoxysilane (Merck).

Fourier Transform Infrared spectroscopy (FTIR) characterization was performed using Nicolet is5 thermo Scientific instrument, Scanning Electron Microscopy (SEM) characterization was performed using FEI Quanta 650 instrument, and absorbance spectra was measured using BEL Engineering Double-Beam UV-M90 Spectrophotometer UV-Vis unit.

3. Methods

3.1 Synthesis of Magnetic Graphene Oxide

The synthesis of the support material was carried out using the method by [11] with some modifications. The amount of 400 mg $FeCl_3 \cdot 6H_2O$ and 211 mg $FeSO_4 \cdot 7H_2O$ were added to the graphene oxide (GO) suspension in water (40mg/mL). The temperature was increased to 85ºC, and ammonia (30% v/v) was added until the pH reached 10. The reaction continued for 45 minutes while stirring. Thereafter, the black solid was centrifuged, washed with demineralized water, and dried at 60ºC.

3.2 Immobilization of Cholesterol Oxidase onto Magnetic Graphene Oxide

Enzyme immobilization was carried out using a method published by[13)] with some modifications. The enzyme is bound to the support material by the cross-linking method. 30 mg of the material was dissolved in 25 mL of ethanol, then 8 mL of (3-Aminopropyl)triethoxysilane was added. The temperature was increased to 50ºC for 8 hours reaction in shaker water bath. The results were centrifuged, washed three times with ethanol and dried at 60ºC. Glutaraldehyde was used as a cross-linking reagent. The amount of 30 mg of magnetic graphene oxide is allowed to react with 5 mL of glutaraldehyde solution for 6 hours at 50ºC. The magnetic material is then separated using a magnet and dried in an oven at 60ºC. Next, the magnetic material was suspended with phosphate buffer (0.01 M, pH 7.4). The enzyme solution was slowly added to the suspension and stirred thoroughly to avoid the bulky form of magnetite. The mixture was incubated at 37ºC for 120 minutes and then the enzymes were separated by a magnet.

3.3 Cholesterol Oxidase Activity Assay

Cholesterol solution preparation is done following a method published by[14)]. Cholesterol solution was prepared by stirring and heating of 500 mg cholesterol in 5 mL Triton X-100. In order to avoid boiling or yellowing of the solution, then 90 mL of distilled water was added slowly for 1 minute until it became cloudy, and then cooled in ice water. By a gentle stirring, 2.07 g of cholic acid was then added to the mixture. The solution was stored at 4ºC until use.

Colour reagent was prepared by dissolving 200 mg phenol and 32 mg 4-aminoantipyrine in 0.01 M phosphate buffer sulfate (pH 7.4). The prepared colour reagents were stored in amber coloured bottles at 4ºC until use. Reagents that are more than a week old are discarded.

The activity assay of cholesterol oxidase was done by adding 1 mL of ChOx solution to 2 mL of cholesterol. The mixture was incubated at 37ºC for 5 minutes. 1 mL of colour reagent and 1 mL of horseradish peroxidase was then added to the mixture and incubated again at 37ºC for 10 minutes. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 500 nm.

3.4 Reusability, Storage Stability and Thermal Stability Test

The reusability of the immobilized enzyme has been done by ten consecutive reaction cycles. After each cycle, the used immobilized enzyme was separated using a magnet and the enzyme activity was observed by enzyme activity assay. The immobilized enzyme was then washed using phosphate buffer (0.01 M, pH 7.4) and reused in a new oxidation cycle. The enzyme activity in the first cycle was considered as initial activity.

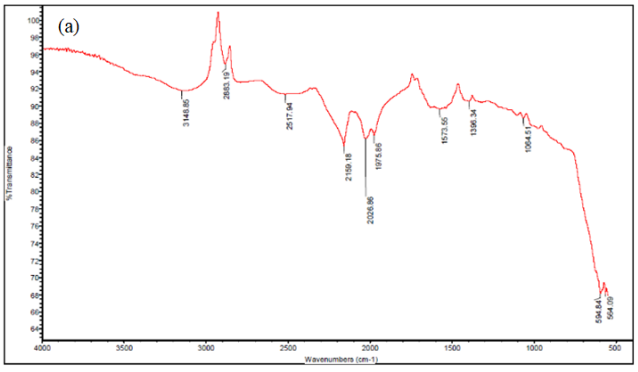
The storage stability of the free and immobilized enzyme was observed by storing both enzymes for 10 days at 4ºC. The activity of the enzymes was observed every day.

The study of thermal stability was carried out by analyzing the enzyme activity at the temperatures of 20ºC, 30ºC, 40ºC, 50ºC, 60ºC, and 70ºC. The immobilized enzyme and free enzyme were both incubated in a water bath at each temperature for 45 minutes. Each enzyme was then cooled at 4ºC for 15 minutes and the enzyme activity was measured using the enzyme activity assay.

4. Results and Discussions

4.1. Characterization of Magnetic Graphene Oxide (MGO) and Immobilized Enzyme (MGO-ChOx)

The results of characterized MGO is shown in Figure 1a. The results of the wavenumber peaks obtained were then compared with previous works of literature which have discussions related to magnetic graphene oxide and cholesterol oxidase to determine their functional groups. The peaks at 564 cm^{-1} and 594 cm^{-1} indicate the presence of Fe-O groups from magnetite, which means that graphene oxide had been successfully magnetized[15]. Several other functional groups derived from MGO material were found at wavelengths of 1064 cm^{-1}, 1396 cm^{-1}, 1573 cm^{-1}, and 3148 cm^{-1}, each of which came from the CN group, C- OH, NH, and OH[12), 15), 16)].



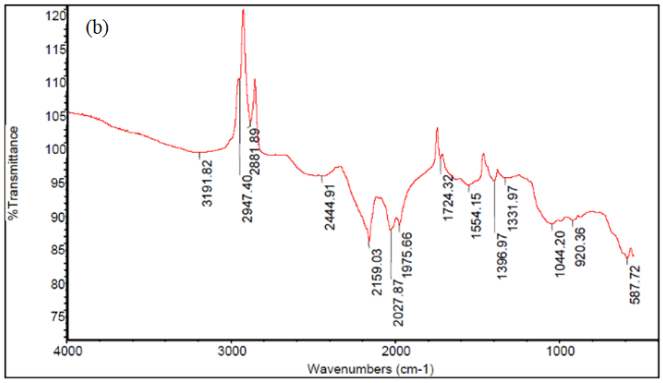


Fig 1. FTIR characterization of (a) MGO and (b) MGO-ChOx

The results of the FTIR characterization for the immobilized enzyme can be seen in Fig. 1b. There are several differences in the results of the FTIR test for the immobilized enzyme, including the peak reading at a wavenumber of 920 cm^{-1} which indicates the presence of a P-O-C group of the FAD cofactor. Another peak reading was found at a wavenumber of 1724 cm^{-1} which was generated by the C=O group of aspartic acid, which is a component of the cholesterol oxidase enzyme[17),18)]. The presence of these two functional groups indicates that the cholesterol oxidase enzyme was successfully bound to the material. In addition, there is also a peak at wavenumber 2947 cm^{-1} which indicates the C-H group of APTES used in the enzyme immobilization process[12)].

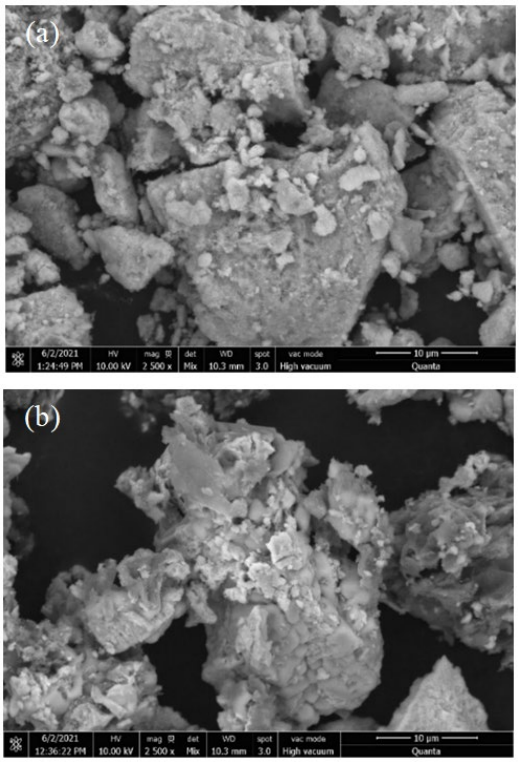


Fig 2. SEM characterization of (a) MGO and (b) MGO-ChOx at 2500x magnification

The SEM image shown from the test results of the MGO material that was not immobilized by the enzyme in Fig. 2a shows solid particles with a relatively flat and non-porous surface. This is different when compared to the SEM results of the immobilized enzyme in Fig. 2b. From the image, it could be seen that the surface of the particles became rougher with a lot of agglomeration. This could be due to the functionalization of the particle surface with APTES and glutaraldehyde prior to the immobilization process, which modified the MGO surface to become more porous. This irregular surface gave the particles higher mechanical integrity[19]. In addition, the roughness of the surface could also be caused by the globular shape of the protein from the enzymes attached to the material[11].

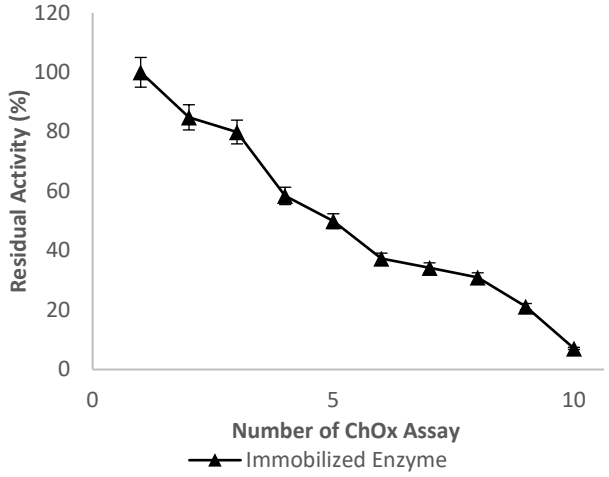


Fig 3. The influence of the number of reuses on the activity of immobilized and free ChOx

Reusability is a significant advantage for the immobilized enzyme, as it can effectively reduce the cost in industrial applications. Figure 3 shows the results of the reuse of the immobilized enzyme after ten repeated oxidation cycles. Enzyme activity decreased after each cycle, with residual activity of 7.10% after the tenth cycle. The results obtained are in accordance with the results of previous studies, it showed that for most enzymes immobilized in solid materials, more than fifty percent of enzyme activity will be lost after five cycles or less[20]. In each cycle carried out, the enzyme activity always decreased. Loss of enzyme activity can occur due to blockage of the active site of the enzyme caused by the accumulation of reaction products on the surface of the immobilized enzyme[13].

The hardest challenge to immobilized the enzyme with the inorganic materials was to control the activity and reusability. The used materials able to recycle and provide more catalytic activity. Somehow, here in this study we succeeded to recycle the materials after oxidation process by ten cycles. However, the performance of reusability still need an improvement to reach the minimum residual activity.

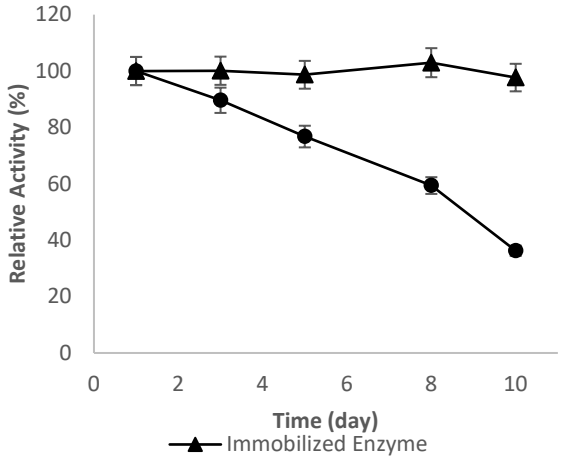


Fig 4. The influence of storage time on the activity of immobilized and free ChOx

The results of the enzyme storage time test are shown in Fig. 4. The immobilized enzyme did not experience a large decrease of activity after ten days, retaining 97.68% of its initial activity, while after the same period the activity of the free enzyme decreased to 36.25%. This indicates that the immobilization of the cholesterol oxidase enzyme on MGO provides an improvement in the storage stability of the enzyme. The cause of the increased stability of the enzyme could be related to the better resistance of the enzyme to conformational changes. In aqueous media, the presence of water can increase the conformational mobility of protein molecules as well as hydrolysis of peptide bonds, which causes the unfolding of enzyme molecules and loss of enzymatic activity[21]. The stabilizing effect exerted by the immobilization of the enzyme on the matrix minimizes the distortion effect that can occur from the aqueous medium at the active site of the enzyme[22].

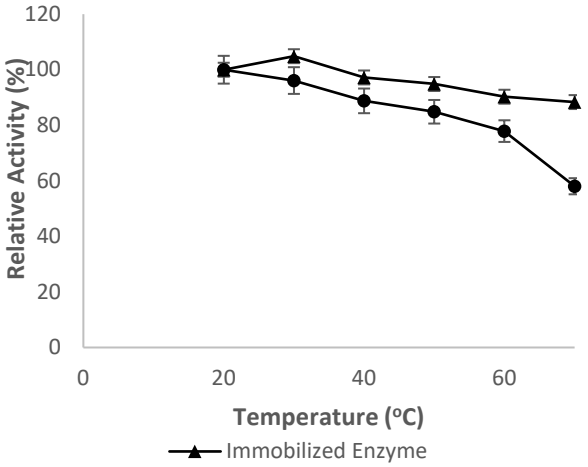


Fig 5. The thermal stability of immobilized and free ChOx

The thermal stability test of the free enzyme and the immobilized enzyme gave the results that can be seen in Fig. 5. The immobilized enzyme still retained more than

90% of its initial activity up to 60ºC and maintained 88% of its activity at 70ºC. Meanwhile, the free enzyme activity was reduced to 88% of the initial activity at 40ºC and had a relative activity of 58% at 70ºC.

The decrease in enzyme activity with increasing temperature can be caused by differences in conformational flexibility between immobilized and free enzymes. This is because the formation of covalent bonds between the enzyme and the matrix on the immobilized enzyme could reduce the conformational flexibility of the enzyme. The decrease in conformational changes caused the rate of enzyme deactivation to decrease[23]. The catalytic performance of the materials are also confirmed due to covalently bonding[24]. A study also confirmed that encapsulated enzyme was able to enhance the final product with a specific characteristic[25,26]. The immobilization of enzyme with a metal materials able to oxidize and enhance the surface area. This kind of method is well performed in numerous research[27,28,29].

5. Conclusion

In this study, magnetic graphene oxide was synthesized and used as a support for the enzyme cholesterol oxidase. Cholesterol oxidase immobilization was successful. In the reusability test, the immobilized cholesterol oxidase enzyme maintained 7.10% of its activity after ten reaction cycles. The cholesterol oxidase enzyme also showed better storage time stability and thermal stability compared to the free cholesterol oxidase enzyme.

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