ORIGINAL ARTICLE

Cholesterol Biosensor Based On Polyaniline Conducting Chitosan Film

Belgis

Technology of Medical Laboratory, Faculty of Vocational, Universitas Airlangga. Jl. Dharmawangsa Dalam 28-30 Surabaya, 60286 Indonesia

ABSTRACT

Introduction: There are various methods in cholesterol analysis, for example, amperometric, enzymatic, and electrochemistry. Those methods are not expressing the suitable methods for cholesterol analysis because of the complicated and long procedure. **Methods:** This experiment is developing cholesterol analysis methods using enzyme immobilization techniques. The enzyme, cholesterol oxidase, immobilized on polyaniline surface conducted chitosan and formed a biosensor. Polyaniline matrix bound to chitosan in a covalent bond, a strong bonding that acts as an enzyme's trap. **Results:** The optimal biosensor has 650 nm maximum wavelength and optimum pH at 7. Immobilization process occurs in 24 hours. The biosensor has 8 minutes response time and can be used one time during 2 weeks active time. The biosensor responses increase linearly at range 0.1 mg/mL and 2.5 mg/mL. The sensitivity at 0.304 mg/ml and accurate value at 89.72%. The detection limit and quantity limit are at 0.00521 mg/mL and 0.0197 mg/mL. **Conclusion:** The result showed that this method can be used as an eco-friendly material for a biosensor. This method can be developed by using the suitable crosslinker for enzyme-surface to enhance biosensor performance.

Keywords: Biosensor, *Cholesterol oxidase*, Polyaniline

Corresponding Author:
Belgis, MSc
Email: belgis@vokasi.unair.ac.id
Tel: +6231 5033869

INTRODUCTION

High cholesterol levels in the blood can increase the risk of various diseases such as hyperthyroidism and anemia (1), nephrosis, militant diabetes, jaundice, malabsorption and myxedema (2). Analysis of cholesterol levels is needed, because of the development of various diseases due to high cholesterol levels. The World Health Organization (WHO) mentioned, the average cholesterol level of adults in the UK who are over 16 years old is 5.5 mmol / L, and that level increases with age. Whereas in China, the total cholesterol rate in people aged 35-64 years is 4.5 mmol / L. WHO estimates that more than 50% of cardiovascular disease and coronary heart disease in developed countries can be associated with blood cholesterol levels of more than 3.8 mmol / L (3)

Referring to the need for a community diagnosis of the determination of cholesterol levels, various instrument designs and clinical detection methods both in the blood and in food are increasingly being developed. Analysis of cholesterol levels in the blood develops from various methods used, including using amperometry, enzymatic and electrochemical (4).

Zhao et al (5) measured cholesterol using modified glassy carbon electrode and encapsulated chitosan matrix. The result is electrodes that are very sensitive to cholesterol, but this method wasn't simple to apply. Meanwhile, Sean et al (6) measured cholesterol in human serum using composite poly (2-hydroxyethyl methacrylate) and the platinum electrode. The enzyme, cholesterol oxidase was immobilized in a hydrogel component. The result really sensitive but it was a complicated procedure.

In previous research, conducting polymers applied to biosensors because it can be used both as immobilization matrices and as redox systems (7–9). Among various conducting polymers, polyaniline is expected to be able to maintain the catalytic ability of enzymes because of their flexibility in chemical structures, natural, and easy to use (7,10–12). Polyaniline (PANi) is being developed as an alternative supporting material for immobilization because the synthesis process is easy, as well as high conductivity and stability (13,14). Chemical polyaniline synthesis can be produced for large quantities. This is very beneficial for the benefit of industrial applications (15,16). Many kinds of research show that polyaniline is known as a material for biosensor development because of its usefulness as an enzyme entrapment matrix and because of its ability as a sensing element material for estimating of various analytes (17). Srivastava et al (18) made biosensor from cholesterol oxidase

which immobilized electrochemically on the surface of polyaniline films to measure cholesterol levels by spectrophotometric methods. The biosensor has response time about 40 s, linearity from 50- 500 mg/dl. This method showed good qualities of biosensor but complicated.

Various enzyme immobilization methods have been used for technological development of biosensor, there are entrapment, modified electrode, electrochemical, physical adsorption, cross-linking, amperometric, and covalent attachment methods (5,19–22). According to the previous research, the covalent binding considered has a strong binding of an enzyme and supporting matrix through covalent bonds. It also has known the advantage of stronger bonding, which is not reversed by pH or ionic strength (19,20,23,24). Therefore, the integration of these two types of materials has been investigated for application in biosensors and controlled drug release (18,25–27).

Chitosan, a copolymer formed by deacetylation of chitin. This polymer possesses hydrogel-like film which is natural, anionic or cationic, biocompatible, biodegradable, and nontoxic polymers (28). The use of chitosan in the application of drug delivery systems is a special interest. As a hydrogel-like material which are biocompatible and non-toxic, chitosan is a medically suitable material as a drug carrier for various drug types (29). In a hydrogel form, chitosan is used on many applications such as cell cultured, wound healing, antibacterial, cosmetic, wastewater treatment and adsorbents, food packaging, separation membrane, and an encapsulated drug delivery system (30–32). In the present work, polyaniline and chitosan films were prepared and applied for cholesterol biosensor using cholesterol oxidase immobilization. The immobilization process by crosslinking method using formaldehyde.

MATERIALS AND METHODS

Samples

Chitosan, 0.75% acetic acid, 37% HCl, aniline (EC: 2005393), ammonium peroxodisulfate, sodium hydroxide, formaldehyde, potassium (I) dihydrogen phosphate, Na2CO3, CuSO45H2O, Na-K tartarat, Folin-Ciocalteau reagent, Bovine Serum Albumin (BSA) standard, cholesterol, cholesterol oxidase from Cellulomonas sp (ChOx) (SIGMA-Aldrich, EC:2328421), control serum, Triton X-100 and distilled water.

Synthesis of polyaniline

Polyaniline synthesized in various aniline concentration, there are 0.5 M, 1 M, 1.5 M, 2 M. Each concentration of aniline was dissolved in 1 M HCl in 10 ml measuring flask for one hour. Each aniline solutions added $(NH_4)_2S_2O_8$ solution and let it formed polyaniline's dope in various time.

Synthesis of polyaniline-chitosan film

Two grams of chitosan dissolved in 80 ml of 0.75% acetic acid and stirred for three hours until the dope is formed (65), 0.01% formaldehyde was added and stirred homogeneously. Chitosan solution molded on a glass surface, formed a thin layer, then washed in aqua dest. Polyaniline's dope coated to chitosan film, formed a chitosan-polyaniline film, then cut it to 5 x 1 cm square.

Immobilize cholesterol oxidase on chitosan-polyaniline film

Polyaniline-chitosan film was sprayed using a formaldehyde solution and allowed to dry. The cholesterol oxidase coated on polyaniline-chitosan film and let it for 24 hours at room temperature.

Enzyme solution preparation

The solutions of ChOx (0.08 ml/100U) added in phosphate buffer (20 mM, pH 70). The cholesterol solution was diluted in 10% Triton X-100 and stored at 4° C.

RESULTS

Biosensor characteristic

Polyaniline synthesized in various aniline concentration and polymerization time. There are 0.5 M, 1 M, 1.5 M, 2 M and 2,3,4, 5 hours various, so it yields 16 difference polymers. The ideal appearance for biosensor should be in bright color, so it could response color changing by pH difference. The polymer that suit for biosensor yielded by 0.5 M in 2 hours polymerization time. It coated on chitosan film and yielded a bright green polymer as shown in Figure 1 and 2. Scanning Electron Microscope (SEM) shown that chitosan-polyaniline's film has pores surface, which the pores will act as an enzyme's trap.

The result of the immobilization process shown by comparing before and after immobilization SEM's picture. As shown in Figure 3, there are clear pores in polyaniline's surface, so there are no enzyme traps on it. Meanwhile, in Figure 4, there is a muddy surface in polyaniline's surface. It is shown that enzyme traps and bond to polyaniline's surface.

The protein content can be measured using the Lowry method. The protein contains before immobilization is

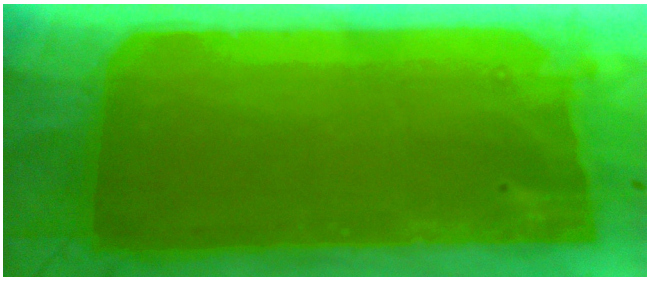

Figure 1: Chitosan-polyaniline film The physic material of biosensor

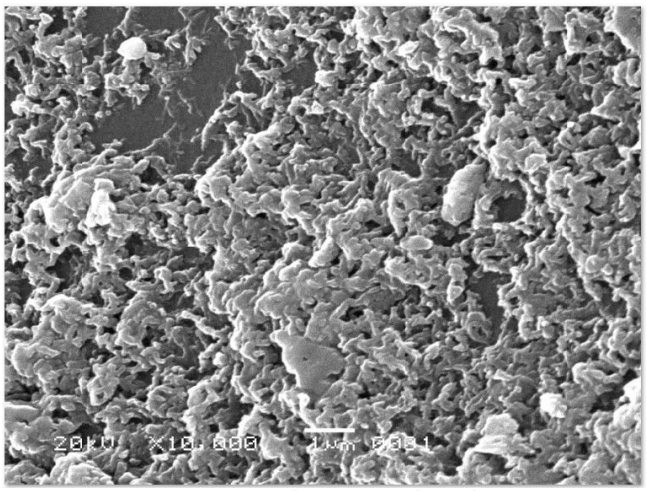

Figure 2: Surface of chitosan-Polyaniline Scanning Electron Microscope (SEM) of the chitosan-polyanilines surface

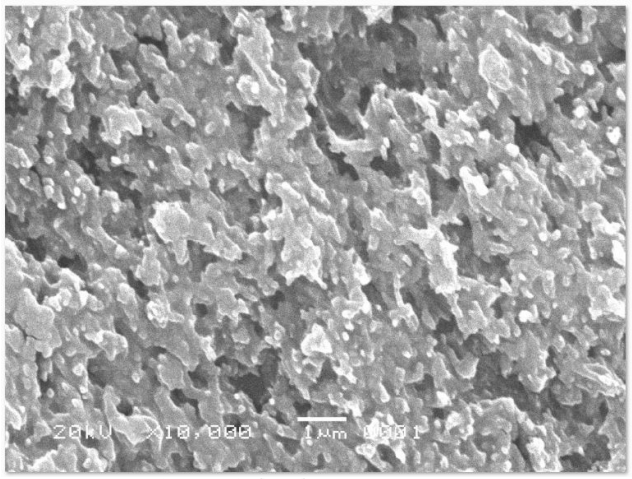

Figure 3: Surface of the biosensor Scanning Electron Microscope (SEM) of biosensors surface

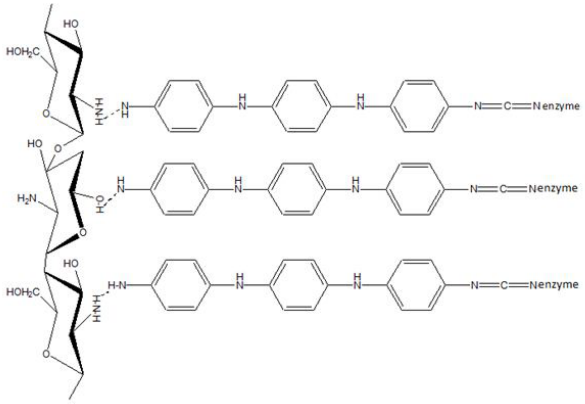

Figure 4: Enzyme-biosensors chain The chemical reaction of cholesterol oxidase and biosensors chain

161.9 µg/ml and after immobilization is 155.4 µg/ml. The difference of protein content shown immobilized enzyme's amount. The immobilization mechanism could be described by Figure 4. The function of formaldehyde is connecting polyaniline surface and enzymes amino acid through N double bond from both sides.

The Fourier Transform Infra-Red Spectroscopy (FT-IR)

Through the FT-IR spectra in Figure 5, the -OH vibration showed at 3435 cm-1, the -NH showed at 1645 cm-1, the -NH2 showed at 1597 cm-1. The saccharide structure showed at 1075 cm-1, it considered as skeletal vibration of C-O, then the C-O-C bridge showed its anti-symmetric stretching at 1155 cm-1. The hydroxyl group showed at 1379 cm-1 as the vibration of the alcohol group in the chitosan. The C-H stretching of chitosan showed at around 2861 cm-1(33).

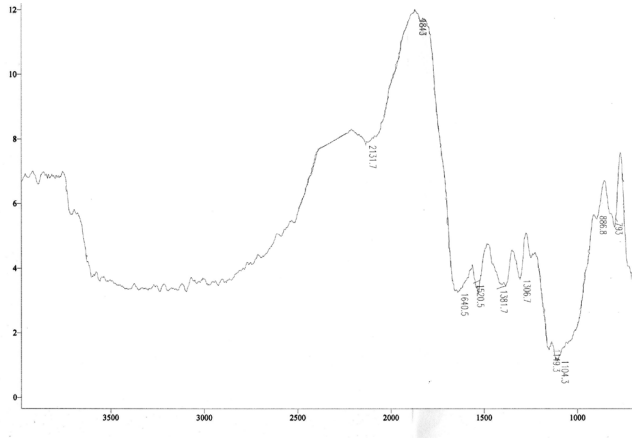

Figure 5: FT-IR Spectra of the biosensor Fourier Transforms Infra-Red Spectra of the biosensor

The maximum wavelength and optimum pH biosensor

The maximum wavelength of the biosensor is determined by measuring biosensor's absorbance using UV-Vis spectrophotometer. The absorbance is measured using various pH at wavelength 380-800 nm with 30 nm intervals. The maximum wavelength of biosensor reached at 650 nm in pH 7. Comparing to polyaniline wavelength at pH 7, there is hipsokromik at 650 nm to 710 nm. The wavelength shift occurs from ϖ electrons states in the direction of the ring ϖ * benzoid (34).

Measuring response time

The response time of biosensor is determined by measuring the absorbance of the cholesterol solution at 20 mg/ml concentration after incubated at 45° C for 15 minutes. The absorbance of cholesterol solution measured in one minute's interval until maximum absorbance.

Biosensor response time is the optimum interaction time between the biosensor and material. This response time determined using 20 mg/ml cholesterol concentration at optimum 45° C for 14 minutes in one-minute intervals. The maximum absorbance is shown biosensor maximum response time, it occurs in 8 minutes incubation.

Biosensor stability

Cholesterol biosensor is a detector that has a lifetime. The lifetime is shown as an absorbance measurement periodically. The stability of biosensor decreased if there

is 30% decreasing absorbance, and biosensor claimed deprived if there is 85% decreasing absorbance (47). The absorbance of biosensor decrease in every measurement because of desorption enzymes.

Data analysis

Cholesterol linear range is measured using standard measurements. It is shown that the higher cholesterol's concentration in the biosensor, the higher its absorbance. Cholesterol's concentration at range 0.1 mg/mL – 2.5 mg/mL has a linear amount to its absorbance. From the experiments shows that there's is 99.4% increasing absorbance because increasing cholesterol concentration.

The sensitivity value determined from the slope in regression equation from cholesterol standard. The amount of sensitivity value is 0.304. It means the absorbance measurement will change by changing 0.304 mg/ml cholesterol's concentration.

Limit of detection measured by equations (1) and (2). The measurement has shown that limit detection of a biosensor is 0.000521 mg/ml. Limit of the quantity measured by equation (3). The measurement has shown that limit of quantity of biosensor is 0.0197 mg/ml.

$$SD = \left\{ \sqrt{\frac{(y_1 - \hat{y}_1)^2}{n-2}} \right\} \quad (1)$$

$$YLOD = A + 3SD \quad (2)$$

$$LOQ = 10 \times LOD \quad (3)$$

The precision value measured by repeating the measurement. The calculation has shown that difference between the first time and the second time measurement is 0.53%. The accuracy of measurement determined as % recovery. The accurate measured using serum concentration The measurement of this biosensor is 89.72%.

DISCUSSION

At 0.5 M aniline concentration and 2 hours polymerization time, the aniline polymer formed a bluish green and was chosen as biosensor material. This is due to the darker color of the polyaniline polymer as concentration increases and polymerization time increases. Biosensors are ideally bright green so they can change color in response to changes in pH.

At neutral pH, polyaniline formed is partially oxidized polyaniline. The reduced polyaniline group consists of two molecules in the form of benzoic rings and amine groups. Whereas the oxidized group of polyaniline consists of a quinoid ring and an imine group. At the level of fully oxidized or at alkaline pH, alkaline polyaniline pernigranilin with a polymer chain consisting only of oxidized groups and physically purple. At a half-oxidized level or at a neutral pH, polyaniline is an emeraldine base and is physically turquoise. And at a partially oxidized and protonated level or at acidic pH, polyaniline in the form of emeraldine salt and is physically green (35).

The chitosan-polyaniline membrane used as a biosensor is ideally bright green so that any change in color due to changes in pH can be responded well. At concentrations of more than 0.5 M and polymerization times of more than 2 hours produce polymers that are darker in color and clot due to longer polymer chains.

The enzyme immobilization process can be carried out by various methods, one of which is using the crosslinking method. The crosslinking method is used to obtain an enzyme predicted firmly bound to the surface of the membrane (36).

The evidence that enzyme has immobilized the membrane is shown by comparing SEM results between the membranes before immobilization and the membrane after immobilization. Immobilized cholesterol levels can be calculated using the Lowry method. The calculation results obtained the levels of enzyme protein before immobilization of 16.19 µg / ml and enzyme protein levels after immobilization of 15.54 µg / ml.

Absorbance was measured using a UV-Vis spectrophotometer at wavelengths of 380-800 nm at intervals of 30 nm. The maximum wavelength is indicated by the maximum absorbance value of the biosensor, and the optimum pH is the pH used when the biosensor shows maximum absorbance. The maximum wavelength of biosensors at pH 6 and 7 is 650 nm, and at pH 8 is 680 nm. The highest absorbance in the biosensor at pH 7, then for the next measurement, the absorbance measurement uses a wavelength of 650 nm and pH 7.

The biosensor response time is the biosensor optimum time when interacting with the analyte. The timing of the biosensor response was carried out at a cholesterol concentration of 20 mg/ml, at the optimum temperature of the cholesterol oxidase enzyme of 45° C for 14 minutes with a difference of one minute. Maximum absorbance achievement at 8 minutes incubation time, so that this time was chosen as biosensor response time.

Lifetime biosensors are shown on absorbance measurements periodically to determine the amount of cholesterol that can be detected by biosensors. The biosensor stability is stated to experience a decrease in ability if there is a decrease in absorbance of 30% in measurements, and biosensors are stated to be unusable if the reduction in measurement reaches 85%. From the measurement results, it is seen that the biosensor has decreased activity at each measurement, so biosensors

can only be used once. The decrease in biosensor measurement ability due to desorption (release of enzymes from the membrane) because the bond between the enzyme and the membrane is less strong.

This research is a preliminary study which needs to be optimized in many methods before implementing for clinical testing. The use chitosan-polyaniline as supporting matrix need to be optimized by varying concentration of the polymer composite methods to produce a compatible membrane. The enzyme bond weakly, so the immobilization method needs further research to produces a good quality of biosensor.

These methods using organic materials that generally have a shorter lifetime than inorganic materials, so this product requires good maintenance so it can be used for a long time and repeatedly.

CONCLUSION

According to the research, it concluded that immobilization of cholesterol oxidase on chitosan-polyaniline film can be used as cholesterol biosensor. The optimum pH of the biosensor at 7, the maximum wavelength of 650 nm, and response time of 8 minutes. The sensitivity value is 0.304 mg/ml, one time maximum stability, detection limit value is 0.00521 mg/ml, the quantity of limit is 0.0197 mg/ml, the accuracy value is 89.72% and precision value is 0.53%. The result showed that this method can be used as an eco-friendly material for a biosensor. This method can be developed by using the suitable crosslinker for enzyme-surface to enhance biosensor performance.

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