ISSN: 1410-8917
Jurnal Kimia Sains & Aplikasi
e-ISSN: 2597-9914

Jurnal Kimia Sains dan Aplikasi 26 (5) (2023): 160-165

Jurnal Kimia Sains dan Aplikasi
Journal of Scientific and Applied Chemistry

Journal homepage: http://ejournal.undip.ac.id/index.php/ksa

Biosensor based on Cellulose Acetate/Glutaraldehyde Membrane Electrodes for detection of organophosphorus pesticides

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https://doi.org/10.14710/jksa.26.5.160-165

Article Info

Article history:

Received: 20th January 2023
Revised: 04th June 2023
Accepted: 22nd June 2023
Online: 31st July 2023

Keywords:
acetylcholinesterase; biosensor; cellulose acetate; organophosphate; potentiometric

Abstract

In recent years, sensor applications have been critical in many fields, especially food safety and pesticides. Organophosphorus pesticides (OPPs) can be detected using a potentiometric biosensor with a membrane electrode made of a new natural material based on cellulose acetate (CA). Acetylcholinesterase was immobilized to 15% modified CA membrane electrodes using glutaraldehyde (GTA) as crosslinking agent and gold (Au) electrode. An indirect method used an acetylthiocholine chloride (ATCl) substrate to find OPPs like chlorpyrifos, profenophos, and diazinon. The working electrode was an CA/GTA membrane electrode, and the reference electrode was an Ag/AgCl electrode, whose potential value was measured with a potentiometer. The surface morphology of the biosensor membrane was investigated using scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM/EDX). It showed that the CA membrane has a smooth, porous surface and is very dense, and its structure consists of 71.27% carbon (C) and 28.73% oxygen (O) with an average diameter of 562.33 nm. A potentiometric biosensor based on AChE inhibition for the detection of OPPs showed a limit of detection (LoD) of 1×10^{-6} µg/L with a linearity range of 1×10^{-6}–1.0 µg/L. The %inhibition value for the chlorpyrifos pesticide was 14.44 to 73.08%, profenophos was 11.98 to 77.98%, and diazinon was 18.58 to 83.27%. Therefore, higher inhibitor concentrations (OPPs) have a greater ability to prevent the AChE enzyme from breaking down the acetylcholine substrate. The biosensor with the CA membrane has a wide linearity range and a low detection limit. The potentiometer rapidly detects pesticide residues.

1. Introduction

Farmers use a variety of pesticides to protect crops and seeds from diseases and pests before and after harvest [1]. The term “pesticide” refers to a toxic chemical that can kill various pests, such as rodents, weeds, insects, and fungi [1, 2]. Pesticides are classified into five groups based on their chemical composition: organochlorine compounds, organophosphorus (OP), carbamates, pyrethrins, and pyrethroids. OP compounds have a wide range of applications as insecticides, nematicides, fungicides, and herbicides and have been used in agricultural pest control for over five decades [2, 3]. The widespread use of organophosphorus pesticides (OPPs) will contaminate foodstuffs, endangering animal and human health [1, 2].

The growing concern about pesticides causing food pollution in agriculture necessitates a concerted effort to identify polluters using dependable, cost-effective, and timely methods. As a result, a wide range of practical and affordable diagnostic tools are now available to rapidly screen for specific pesticide residues in food and the environment [4, 5, 6]. Methods for determining pesticide residues generally use HPLC, LC-MS/MS, and GC-MS/MS [7, 8, 9]. Although this method has a high level of

sensitivity, it is costly, time-consuming, and requires a substantial amount of reagent samples [10]. Due to these limitations, an alternative methodology has been proposed in recent years: developing biosensor methods to detect pesticides [11, 12].

Biosensors are devices integrating biological elements (e.g., enzymes, DNA probes, antibodies) for the detection of analytes (e.g., enzyme substrates, complementary DNA, antigens) and transduction elements used to convert signals of analyte interactions with bio-receptors into electronics [1]. Based on signal transduction techniques, biosensors are classified into electrochemical, optical, piezoelectric, and mechanical. Electrochemical enzyme-based biosensors have been widely utilized to detect pesticide compounds rapidly because of their minimal pre-treatment, brief analysis, and high sensitivity and selectivity [13, 14, 15]. In addition, it is low cost, simple design, and small size, making it an excellent method for developing portable biosensors [16].

Bio-sensing can identify OP compounds by measuring the activity of residual enzymes, such as acetylcholinesterase (AChE), a hydrolase enzyme that plays an essential role in the central nervous system (CNS) and whose inactivity can result in respiratory system disorders, paralysis, and death [17]. All OP compounds are anti-AChE and work through the general mechanism of phosphorylation of AChE. The OP compound disables AChE to catalyze acetylcholine in synaptic membranes into choline, along with the excessive formation of acetate enzymes that cause cholinergic toxicity [18]. Inhibitors of OP compounds form a covalent bond with serine located on the active site of AChE, resulting in enzyme inactivation [19, 20, 21]. The intensity of AChE inhibition is proportional to the concentration of the OP compounds and is used as the principle of the OP compound concentration detection method [19, 20, 21, 22].

Membrane materials for biosensor electrodes must have several properties, including good mechanical properties, thermal stability, and chemical resistance. Cellulose acetate (CA) has recently been studied for its outstanding thermal stability, chemical resistance, biocompatibility, and biodegradability [12]. It can be used for various applications (e.g., films, membranes, or fibers). Recent trends in CA applications applicable to surface device diagnostic kits and sensing materials have been reviewed in this section. According to the literature review, CA can achieve at least three sensing approaches, including optical, colorimetric, and electrochemical methods.

The CA is only used as a supporting membrane for immobilizing biological elements in electrochemical methods (i.e., enzymes, antibodies, and aptamers). Before enzyme immobilization, CA is usually activated. CA modifications can improve attachment to biological elements or sensing processes through various mechanisms. Crosslinking agents are the most common in CA activation among the different methods. Crosslinking agents containing glutaraldehyde (GTA) can activate membrane CA. GTA is also used to create new bioelectronic devices with high stability and selectivity [23]. The enzymes, such as AChE, can then be immobilized on activated CA membranes [16].

A working electrode made of inert Au wire covered with CA and GTA membranes was used in this process. The GTA as a crosslinker with CA was based on forming a bond between the CA functional group and the carbonyl group of GTA. The interaction of CA with GTA occurred through covalent bonds. The crosslinker concentration dramatically affects the formation of the electrode membrane, research by Koseoglu-Imer *et al.* [22], Aburabie *et al.* [23], Wu *et al.* [24], and Kumar and Sundramoorthy [25] showed that 25% GTA concentration is stable. Using the crosslinking agent GTA, the cellulose acetate membrane produces OH groups, C-H stretching, and C-O-H and C-O-C asymmetric stretching. The –OH and –C–O–C groups increase in intensity as the crosslinking reaction increases. Based on its structure, GTA has two reactive aldehyde groups. The aldehyde group is very reactive to the hydroxyl group in CA, so the aldehyde group will form a covalent bond with the hydroxyl group. The compound will form a layer or matrix in which the enzyme molecules on the surface of the electrode will be trapped because of how the structure is completed. One of the advantages of using the immobilization method is that the enzyme is electrostatically bound to be stable with the transducer.

A pesticide biosensor based on the AChE enzyme immobilized CA and GTA membrane as a crosslinker. The success factor is the ability of an enzyme to bind to the surface of the biosensor membrane and remain active during application [26]. This study discusses using an AChE enzyme-based biosensor connected to a simple potentiometer transducer to detect OPPs based on the measurement of %inhibition of enzyme performance and electrode response time to produce electrodes with optimal electrode performance.

2. Methodology

2.1. Materials and Instrumentations

Acetylcholinesterase (AChE) 1.17 mg with activity of 425.94 units per mg (EC. 3.1.1.7), acetylthiocholine chloride (ATCl), cellulose acetate (CA), glutaraldehyde (GTA), potassium chloride, chlorpyrifos, profenophos, and diazinon pesticide were bought from Sigma Aldrich (St. Louis, MI, USA). Ethanol, acetone, and phosphate buffer solution (PBS) pH of 8.0 were prepared by mixing standard stock solutions of Na_2HPO_4 and NaH_2PO_4 obtained from Merck (Darmstadt, Germany). The potentiometer is the instrument used to measure the potential values. For electrochemical biosensors, a gold (Au) electrode was used as a working electrode, a platinum (Pt) as a catalyst to accelerate the electrolysis process, and an Ag/AgCl as a reference electrode. SEM- EDX-mapping using Phenom Desktop ProXL at the Terpadu Laboratory University of Islam Indonesia.

2.2. Process of Ag/AgCl Electrolysis

The Ag/AgCl standard electrode was created by electrolyzing Ag wire in a 0.1 M KCl solution for

25 minutes. The thickness of AgCl on the Ag wire was affected by the electrolysis time. Furthermore, the formed Ag/AgCl wire was allowed to dry in the open air. As an Ag/AgCl comparison electrode, an Ag/AgCl wire was then inserted into the electrode body.

2.3. Preparation of Enzyme-immobilized Membranes

The Au electrode was dipped in a CA membrane solution with a concentration of 15% (w/v). Once the CA layer was formed, the electrodes were rinsed with distilled water three times. The CA membrane-coated part of the Au electrode was soaked in a 25% GTA solution for 6 hours. Furthermore, electrodes were rinsed with distilled water and PBS (pH 8), then formed into an electrode membrane (EM). Phosphate buffer helped to maintain the optimum pH, allowing enzymes to continue functioning optimally. The EM was then incubated in the enzyme AChE for 48 hours at 4°C. Unused EM was stored in PBS solution (pH 8.0) at 4°C. The measurement components, such as standard electrodes, working electrodes, ATCl substrates, and inhibitor solutions, must be kept for approximately 2 hours at ambient temperature before measuring the biosensor response. The condition of the component was stable and capable of producing a good response.

2.4. Enzyme Inhibition Measurements

The inhibition percentage (%I) represents the pesticide's (the inhibitor's) ability to inhibit enzyme performance. After interacting with the inhibitor, the inhibited enzyme rate correlated with the inhibitor concentration and interaction time (incubation time, 20 ± 1 minutes). As a result, the enzyme's activity was inversely proportional to the inhibitor concentration. Potential measurement using potentiometric methods was conducted to determine enzyme inhibition for pesticide detection. After the biosensor electrode had stabilized, the potential value in the presence of ATCl substrate (1×10^{-3} M) was measured, denoted as E_0. The enzyme activity was then inhibited by adding a known variation of pesticide concentration, and the potential value (E_1) was measured, which was proportional to the inhibitor concentration in the solution. Eq. (1) shows the formula to calculate the inhibition percentage (%I).

$$\%\,I = \frac{E_0 - E_1}{E_0} \times 100\% \quad (1)$$

where, E_0 is the initial potential value, and E_1 is the potential value after enzyme inhibition.

3. Results and Discussion

3.1. Component of Biosensors

This study used CA as a membrane electrode because it is stable and has a mechanical strength that can hold small materials. The potential value of the enzyme-catalyzed reaction against the analyte determines by the medium, which is the electrode membrane. The performance of the biosensor depends on the compositional parameters of the Au electrode membrane. When making CA membranes through the phase inversion method using instantaneous liquid-liquid demising, a polymer base material was changed from the liquid phase to the solid phase by removing the solvent with a solvent that has different properties. The solvent that coats the CA membrane was removed using distilled water to obtain a porous CA membrane. Then, the GTA crosslinking method was used to stop the AChE enzyme from moving around. The function of GTA was as a bifunctional reagent between CA and enzymes [27, 28].

The biosensor consists of a receptor (biological recognition element), a transducer, and a signal detector (Figure 1). Biological recognition elements (enzymes) must be analyte-specific to detect analytes in different samples accurately. Due to the near position proximity of the recognition elements and analytes, chemical changes are likely to occur, such as the formation of electroactive species, reduction in the form of by-products, and consumption of oxygen [29]. Chemical changes are detected through the transducer, i.e., potentiometric, and displayed on the control system.

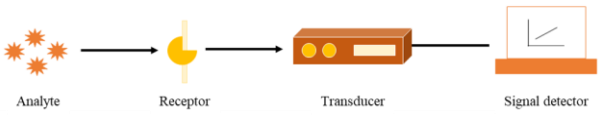

Figure 1. The basic components of an electrochemical biosensor

In this study, an AChE enzyme-based OPP biosensor has been designed. OPPs work mostly by stopping the AChE enzyme from doing its job, which causes problems with how the CNS works. AChE is the primary CNS enzyme that catalyzes the hydrolysis of the neurotransmitter acetylcholine into choline and the enzyme acetate formation. In the case of AChE inhibition-based OP biosensors, the resulting signal is inversely proportional to the concentration of the OP compound. In other words, an increase in the attention of the OP compound causes a weak signal. AChE biosensor works on the inhibitory effect, where AChE is used as a biorecognition element to detect toxic organophosphates [20, 30]. If inhibitors (OPPs) are absent in the analyte, acetylthiocholine is converted to thiocholine and acetic acid. However, if the inhibitor is present in the analyte, the thiocholine concentration decreases, or no thiocholine and acetic acid are produced. In other words, it is completely inhibited [31].

3.2. Characterizations of CA/GTA Membrane

SEM microscopy (Figure 2) revealed several important aspects regarding changes in membrane surface morphology. CA membranes with GTA as a crosslinker presented low surface porosity due to the crosslinking effect caused by covalent interactions between aldehyde and hydroxyl groups on the membrane surface. In the CA and GTA membrane functionalization phase, AChE enzyme immobilization showed the presence of clumps on the membrane surface. Figure 3a, an analysis of membrane morphology, shows a mean pore size diameter of 562.33 nm. Figure 3b, clearly defined for the C and O atoms, identifies the atomic dispersion and the non-porous and smooth membrane surface. Elements C and O were detected in the EDX element analysis (Figure 3c), indicating CA/GTA fibers within the membrane.

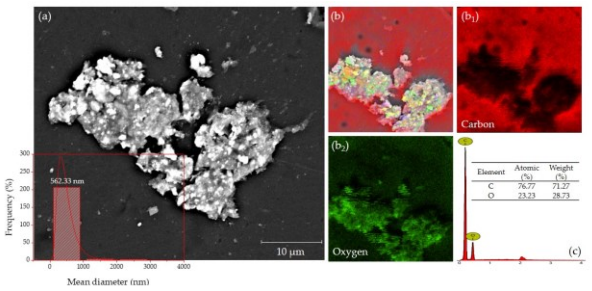

Figure 2. Characterizations of CA/GTA membrane after AChE enzyme immobilization by using (a) SEM, (b) SEM-mapping of (b_1) carbon and (b_2) oxygen, (c) SEM- EDX

3.3. Inhibition of Enzymatic Biosensor

The inhibition percentage (%I) represents the pesticide's (the inhibitor's) ability to inhibit enzyme performance. Pesticide biosensor designs typically rely on quantitative enzyme activity measurements before and after contact with the substrate. The %I produced after interacting with the inhibitor will be proportional to the inhibitor concentration and interaction time (incubation time). As a result, the activity of the enzyme residue is inversely related to the concentration of the inhibitor. The biosensor responds to the AChE enzyme reaction due to contact with the ATCl substrate, determined by the resulting product concentration on the enzyme surface. If the enzyme activity increases, it is not entirely replaced by a transfer in the substrate due to the diffusion process. Therefore, only one of the enzyme's active sites can interact with an acetylcholine substrate. The presence of an inhibitor influences the sensitivity of the immobilized enzyme to inactivity [30].

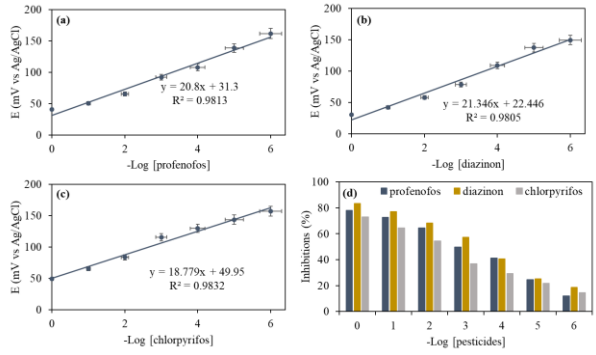

Figure 3. Calibration curves for (a) profenofos, (b) diazinon, and (c) chlorpyrifos in PBS (pH 8.0) at an ATCl concentration of 10^{-3} M, (d) Inhibitions for various pesticides using EM CA/GTA

Linear ranges and LOD of biosensors were analyzed for the pesticides chlorpyrifos, profenofos, and diazinon with a potentiometer. Linear relationships were obtained for E (mV vs. Ag/AgCl) vs. OPP concentrations in the concentration range 1×10^{-6} to 1.0 µg/L with coefficients of 0.9832, 0.9813, and 0.9805, respectively (Figure 3a-c). An increase in pesticide concentration causes a decrease in potential value due to inhibiting enzyme activity. LOD was obtained from the 1×10^{-6} µg/L biosensor.

The administration of inhibitors can affect the enzyme activity and the concentration of the resulting product, so the potential value is small. The percentage inhibition curve shows the results of biosensor analysis with CA and GTA membranes. The curve shows the relationship between the percentage of inhibition (%I) to −log [OPPs] at a substrate concentration of 1×10^{-3} M from the lowest measurement of profenophos inhibitor concentration (1×10^{-6} µg/L) to the highest concentration (1.0 µg/L) (Figure 3d). The %inhibition value for the chlorpyrifos pesticide was 14.44 to 73.08%, profenophos was 11.98 to 77.98%, and diazinon was 18.58 to 83.27% (Figure 3d). Therefore, it can be assumed that the higher the inhibitor concentration (OPPs), the higher the inhibitor's ability to inhibit the AChE enzyme activity in hydrolyzing the acetylcholine substrate [31]. The capability of the inhibitors to limit enzyme activity shows that the Au/CA/GTA@AChE electrodes function admirably in pesticide detection.

3.4. Response Time of Enzymatic Biosensor

Due to the equilibrium of the electrode membrane reaction with the analyte testing, a response time is needed for the electrode to give a constant response. When making a biosensor, it is essential to consider how fast electrons move from the enzyme's surface to the electrode's surface [26]. This transfer should be quick to provide an accurate measurement response. Changing the concentration of inhibitors (OPPs) was used to determine how long coated wire electrodes took to respond. The potential value of each inhibitor concentration was measured every 1 to 10 minutes to obtain a constant potential from low to high concentrations. The response time given by the biosensor electrode to pesticides was 5 minutes (Figure 4). The average response time for the potentiometric biosensor was 5 minutes, and the analysis results were the same for all different concentrations of the pesticide inhibitor. Several studies have shown a fast response, as in the research by Zhang *et al.* [32] on the Prussian blue nanocubes/reduced graphene oxide electrode membrane with the AChE enzyme to detect organophosphate pesticides [33]. It gave a faster response of 10.9 seconds. Even though this study delivered an average response of 5 minutes, it showed that the biosensor with a potentiometer circuit had a stable potential value at 5 minutes. This biosensor type is suitable for field monitoring because it is easy to use and gives quick results.

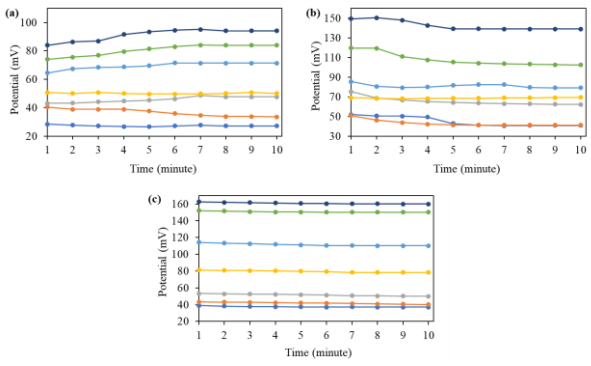

Figure 4. Response time of enzymatic biosensor (a) profenofos, (b) diazinon, and (c) chlorpyrifos

4. Conclusion

For organophosphate compounds like chlorpyrifos, profenophos, and diazinon, potentiometric biosensors can be made by crosslinking the AChE enzyme on the Au–CA surface electrode membrane with GTA. Overall, it can be concluded that for inhibitor concentrations of 1×10^{-6}– 1.0 µg/L, SEM revealed the morphology of the CA/GTA membranes to have smooth and agglomerated surfaces. As an OPP detector, the electrode membrane biosensor is based on a potentiometric approach, a significant inhibition value depending on the concentration of the inhibitor (pesticide), and a response time of 5 minutes. The results show that these biosensors could be used as a promising analytical tool to test for traces of pesticides in food crops.

Acknowledgment

The authors gratefully acknowledge the support of the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia in 2021–2022 as part of an applied research scheme. The authors thank the team for contributing to this research and the University of Halu Oleo (UHO). Terpadu Laboratory of the University of Islam Indonesia (UII) for SEM-EDX analysis.

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