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A Novel Enzyme Biosensor Based on Ag/Reduced Graphene Oxide/Chitosan Membrane with Potentiometer for Pesticide Detection

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ABSTRACT: Long-term accumulation of pesticides in the environment to human and animal health. Acetylcholinesterase (AChE) biosensors with highly sensitive potentiometer transducers based on the membranes of Ag, reduced graphene oxide (rGO), and chitosan (CS) has been successfully developed. The membrane was made with a composition of 0.5 mM $AgNO_3$, 2.5 mg/mL rGO, and 2% (w/v) CS coated on the surface of the Au electrode. The composition of the membrane with three ratios, namely 1:1:2, 2:1:3, and 3:1:4. Then, membrane Ag/rGO/CS and the enzyme AChE were immobilized on the membrane surface. The prepared biosensor has excellent conductivity, catalytic activity, and biocompatibility attributed to the synergistic effect of Ag/rGO/CS and glutaraldehyde (GTA) as crosslinkers and providing a hydrophilic surface for AChE adhesion. The linear range in biosensors is 1×10^{-8} to 1 μg L^{-1} with a regression coefficient of 0.9803 for 1:1:2 membrane, 0.9836 for 2:1:3 membrane, and 0.9850 for 3:1:4 membrane. The LOD is about 1×10^{-7} μg L^{-1} for all membranes. In addition, the biosensor showed good sensitivity, acceptable reproducibility, and stability, having an RSD of less than 5%. This biosensor makes it possible to provide a new and promising tool for analyzing pesticides, especially organophosphates.

Keywords: Acetylcholinesterase, Ag nanoparticles, chitosan, pesticide, reduced graphene oxide

INTRODUCTION

Currently, the productivity of agricultural products has decreased due to attacks by plant pest organisms (PPO), which will reduce the income of Indonesian people who depend on this sector (Salim et al., 2020). The impact of pest attacks also impacts state losses, so efforts are made to use pesticides that can fight pest attacks to increase the productivity of agricultural products. Organophosphates are one of the pesticide groups that are widely used by the community (Van Dyk & Pletschke, 2011). However, the use of pesticides has the potential to harm the environment and human health due to their nature which is difficult to decompose, so they become toxic substances (Kaur et al., 2013; Sahub et al., 2018). Therefore, the Environmental Protection Agency (EPA) took steps to solve this problem by detecting pesticides (Kumar & Melo, 2017).

Pesticide detection generally uses analytical techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) (Almeida et al., 2020; Huang et al., 2018; Ramadhaningtyas & Aryana, 2019; Song et al., 2019). However, this method is costly, time-consuming, and requires special skills. Biosensors are an alternative method that has become popular and is of research interest. As technology and science develop, researchers have tried to develop and innovate to improve the performance of biosensors, especially the sensitivity and stability values (Kumar & Melo, 2017).

Detection of organophosphate pesticides using an acetylcholinesterase (AChE) enzyme-based biosensor. The maximum performance of the biosensor needs to pay attention to several things: namely, the enzyme activity needs to be maintained, and the selectivity and the electron transfer process between the enzyme and the electrode need to be considered so that the enzyme does not inhibit the process. Therefore, several materials are needed to improve the

performance of biosensors with good electrical conductivity and high biocompatibility, as well as materials that have good enzyme immobilization ability (Kim et al., 2015; Prokhorov et al., 2016; Salehi et al., 2016). Materials that are widely used as supports for biosensors are nanomaterials because they have unique physical and chemical properties (such as size, composition, magnetic properties, conductivity, and mechanical strength) (Du et al., 2012; Walcarius et al., 2013; Zhang et al., 2014).

Ag nanoparticles (NP) are one of the nanoparticles that have been proven to be suitable conductive materials among other metals because they have an excellent electrocatalytic response so that they can facilitate the electron transfer process to increase the sensitivity value of the biosensor (Naghib et al., 2018). In addition, Ag NPs can provide a microenvironment to maintain biomolecular bioactivity (Zhang et al., 2019) and more efficient electron transfer between immobilized biomolecules and the electrode substrate. Therefore, Ag NPs can become critical to the analytical performance of biosensors. Reduced graphene oxide (rGO) is one of the nanomaterials developed to support performance of biosensors. The rGO is used in electroanalysis because it has a large surface area, good electronic transport properties, and high electrocatalytic activity (da Silva et al., 2018; Rahayu et al., 2020). In addition to rGO, chitosan (CS), which is a polysaccharide biopolymer from the deacetylation of chitin compounds, is widely used in electroanalysis because it has strong binding properties, is a non-toxic material, has muscular mechanical strength, is easily available, and also has good adsorption ability (H. Zhao et al., 2015; Zhou et al., 2017). Furthermore, CS is a suitable matrix for helping enzyme immobilization (Hermanto et al., 2020). In this study, we used Ag/rGO/CS as a membrane on the biosensor electrode to obtain good sensitivity and a low detection limit (LOD) to detect pesticide residues.

EXPERIMENTAL SECTION

Materials

Acetylcholinesterase (AChE, EC 3.1.1.7, C3889-500UN), acetylthiocholine chloride (ATCl, A5626), glutaraldehyde (GTA), sodium citrate and CS from shrimp shells, ≥95% (deacetylated), diazinon pesticides were purchased from Sigma-Aldrich, St. Louis, MI, USA. Silver nitrate, graphite, urea, sodium hydroxide, potassium chloride, citric acid, acetic acid, ethyl alcohol, potassium permanganate, hydrogen peroxide, and phosphate buffer solutions (PBS) pH 8.0 were purchased from Merck, Darmstadt, Germany.

Apparatus

The potential value of pesticide detection is measured using a potentiometer (SANFIX DM-888C) as a transducer with an Au wire electrode coated with Ag/rGO/CS membrane as the working electrode and Ag/AgCl as the reference electrode. Electrolysis of Ag/AgCl uses Pt wire as a cathode and Ag wire as an anode connected to a battery as a source of electric current. Morphology or surface structure and elemental analysis or chemical characteristics of membrane modification were studied using a Phenom Desktop ProXL SEM-EDX, and X-ray diffraction (XRD) analysis of the prepared samples was performed using a Bruker D2 Phaser with Cu. Radiation of K (= 1,541). The Fourier Transform Infrared (FTIR) analysis wwa carried out a Shimadzu type IRPrestige-21.

Synthesis of Graphene Oxide

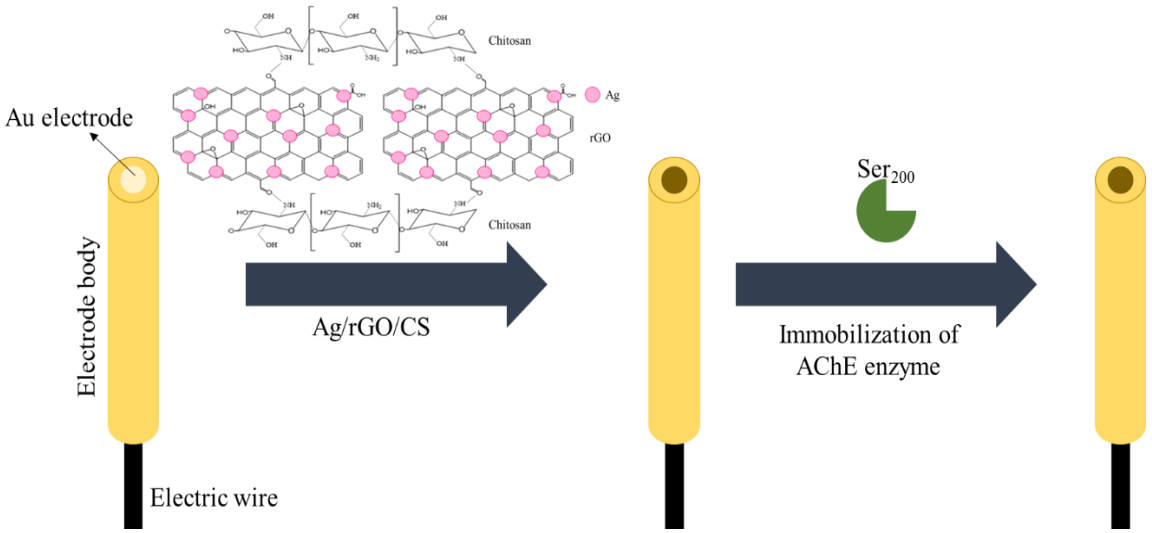
Graphene oxide (GO) was prepared from graphite powder according to the modified Hummers' method (Hummers & Offeman, 1958; P. Zhang et al., 2019). Weigh 2 g of graphite and 4 g of $NaNO_3$ into the flask, then add 98 mL of concentrated H_2SO_4. The mixture was stirred in an ice bath for 4 hours. During the moving process, slowly add 8 g of $KMnO_4$ to the mixture after 1 hour of heating (25°C). The mixture was stirred for 2 hours at 25°C. Then, 200 mL of double distilled water and 15 mL of 30% H_2O_2 were added gradually while stirring, and the temperature was adjusted to 90 °C to form a yellow suspension. The yellow suspension was then centrifuged, and the precipitate obtained was washed with double-distilled water until the pH of the filtrate was neutral and dried in a vacuum at 60°C.

Synthesis of Reduced Graphene Oxide

The reduced graphene oxide (rGO) was carried out by adding 2 g of GO to 100 mL of double distilled water. Then, add 4 g of urea, where urea acts as a reducing agent and N doping and hydrothermally at a temperature of 160 °C for 12 hours. Finally, the black solid was collected by centrifugation, washed with double-distilled water several times, and dried in a vacuum at 105°C overnight. The black powder obtained was dried at 1000 °C for 1 hour.

Preparation of Ag/rGO/CS Membrane

The manufacture of Ag/rGO/CS membranes from the modified method (Naghib et al., 2018). The membrane was made with 3 different ratios of 0.5 mM $AgNO_3$ components, while the rGO components were 2.5 mg mL^{-1} and 2% (w/v) CS was constant. The mixture of $AgNO_3$ and rGO was homogenized using a vortex and then ultrasonically processed. This process is carried out until the dispersion turns grey. The Ag/rGO mixture was then gradually added to the CS solution and 4 M NaOH 10 μL and stirred until the solution formed a paste. The ratios of Ag/rGO/CS membrane composition are as follows: 1:1:2; 2:1:3 and 3:1:4.



Scheme 1. Schematic representation of the preparation process for the Ag/rGO/CS membrane electrode biosensor immobilized by AChE enzyme.

Preparation of Ag/rGO/CS@AChE Electrode

Preparation of biosensor membrane electrodes was presented in the **Scheme 1**. The electrode design of the coated wire type biosensor has an electrode body made of plastic measuring 7 cm with a diameter of 1 mm. Then, the Au electrode with a length of 1.5 cm and a diameter of 0.4 mm was connected to a Cu electric wire and soldered using tin. The electrodes that have been made were coated with Ag/rGO/CS membrane and then immersed in 25% GTA solution for 6 hours at room temperature. The electrodes were rinsed with double-distilled water and PBS pH 8.0. The Ag/rGO/CS membrane electrodes were then immobilized in the AChE enzyme for 2 × 24 hours at 4 °C. Then, the electrodes are dried at 4 °C and stored at 4 °C when not in use.

Measurement Procedure

AChE biosensor measurement using a potentiometer transducer. The biosensor substrate was detected in an ATCl solution with a 1×10^{-3} M concentration. Then, the potential value was recorded every 1 min interval until it was constant. The potential value obtained is then used as the substrate potential. Before measurements, the Ag/rGO/CS@AChE membrane electrode biosensor was washed with PBS (pH 8.0) three times to remove loosely adsorbed AChE. The reference electrode uses an Ag/AgCl electrode.

For the measurements of potential pesticide value, the pesticide sensing performance was evaluated by incubating the Ag/rGO/CS@AChE electrode into a solution of different concentrations of diazinon pesticide for 25 minutes. Then, the electrode was washed with double-distilled water and PBS pH 8.0 before continuing to measure the potential value into a 1×10^{-3} M ATCl substrate solution. Then the ATCl potential value was measured as described above. At all pesticide concentrations, use the same method. The percentage inhibition (I %) of the pesticide on the enzyme electrode, which was taken as a sensing signal for the pesticide diazinon, was obtained by the following formula:

$$\%I = \frac{E_0 - E_1}{E_0} \times 100\%$$

Where, E_0 is the ATCl potential in the biosensor without pesticides and E_1 is the ATCl potential in the biosensor with pesticide inhibition.

RESULTS AND DISCUSSION

Characterizations of Ag/rGO/CS Membrane

The FTIR spectrum of the Ag/rGO/CS (3:1:4) membrane is shown in **Figure 1**(**a**). The presence of characteristic peaks of CS at 1070.49, 1581.63, and 1658.78 cm^{-1}, respectively, represent the C–O, N–H and C=O functional groups, as well as the specific group of rGO, namely the C=group. Aromatic C=C is indicated at the absorption level of 1390.68 cm^{-1}, and the broadband around 3431.36 cm^{-1} is the absorption of the O-H group associated with the hydroxyl, carbonyl, carboxyl, epoxy, and ester groups. It proves the presence of various functional groups containing oxygen in the samples of rGO and CS. The characteristic peak for the O–H absorption region indicates the presence of intermolecular bonds from one component to another. The characteristic signal of the N−H bending of the secondary amide shift from 1593.20 to 1581.63 cm^{-1} indicates the presence of newly formed amide bonds between rGO and CS. It can also be seen that the peak of 2931.80 cm^{-1} is the vibrational peak of C–H, which indicates that the membrane is rich in interactions between the CS matrix and Ag nanoparticles (Căprărescu et al., 2020). The shift of several peaks occurs due to coordination bonds between Ag atoms and electron-rich oxygen or nitrogen groups (An et al., 2018).

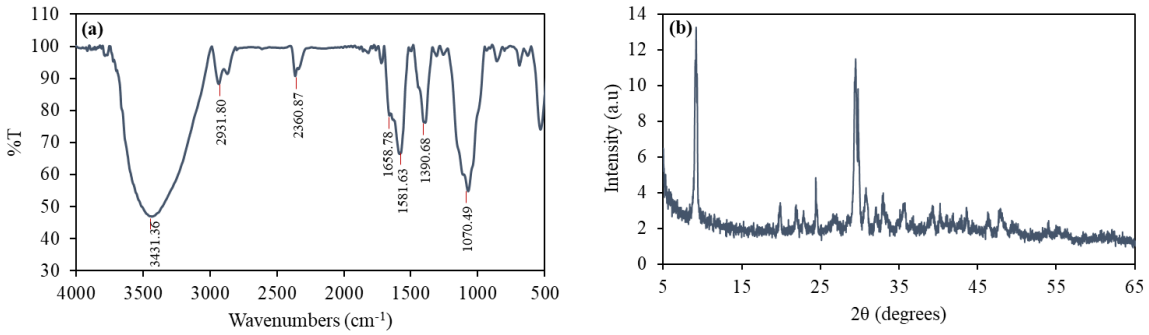


Figure 1. Characterizations of Ag/rGO/CS (3:1:4) membrane (**a**) FTIR and (**b**) XRD

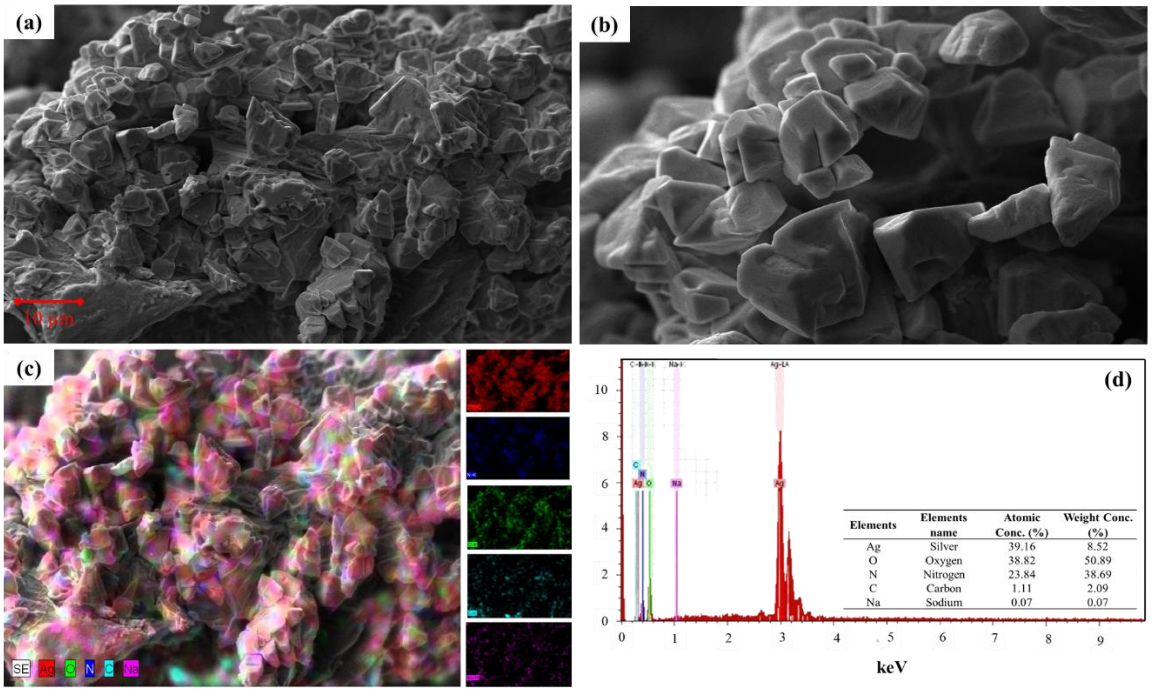


Figure 2. SEM-EDX-Mapping characterizations of Ag/rGO/CS (3:1:4) membrane SEM of (**a**) 1000× and (**b**) 3000×, (**c**) SEM-elemental mapping and (**d**) EDX of Ag/rGO/CS membrane

According to research conducted by Setiadji et al., (2018), it is known that rGO has a peak of 2θ in the range of 24-28°. The presence of rGO iis indicated by the appearance of a sharp peaks at 29.521° (d-spacing 3.02338 Å),29.833° (d-spacing 2.99247 Å), and 24.508° (d-spacing 3.62922 Å) in the diffractogram shown in **Figure 1**(**b**). These results are based on the results of research by (Setiadji et al., 2018). Meanwhile the appearance of peaks at 39.296°, 43.632°, and 46.392° with successive d-spacings of 2.29090, 2.07279, and 1.95569 Å. So it can be determined that the sample is an Ag/rGO/CS.

The diffractogram shows a diffraction peak at 9.229° with a d-spacing of 9.57467 Å, which indicates the presence of GO. The change in d-spacing (distance between layers) from GO of 9.57467 to 3.02338 Å and 2.99247 Å in rGO means that the GO reduction process has been running well. Based on the peak diffractogram, the resulting rGO has a crystalline phase. The difference in peak diffractogram for amorphous and crystalline phases can be seen from the shape, whether the peak is sharp or broad. From **Figure 1**(**b**), the peak formed is sharp, indicating the formation of crystals on the Ag/rGO/CS membrane without immobilizing the AChE enzyme. The sharpness of the resulting peak shows high crystallinity properties (Setiadji et al., 2018).

The surface morphology of the samples was observed in SEM (**Figures 2**(**a**) and (**b**)), which provide information on interfacial interaction. The membrane formed is composed of nano-sized particles, and rGO looks compact, very dense, and well distributed in the CS polymer matrix. Compact, dense morphology and prominent, and rough edges on the membrane surface will result in an increased contact area and better electron transfer properties. It is due to the conductivity properties of rGO and some Ag

nanoparticles, which are well distributed on the membrane surface and will initiate the electron transfer process. Ag and rGO nanoparticles are reported to increase the membrane surface area and electron transfer (Naghib et al., 2018).

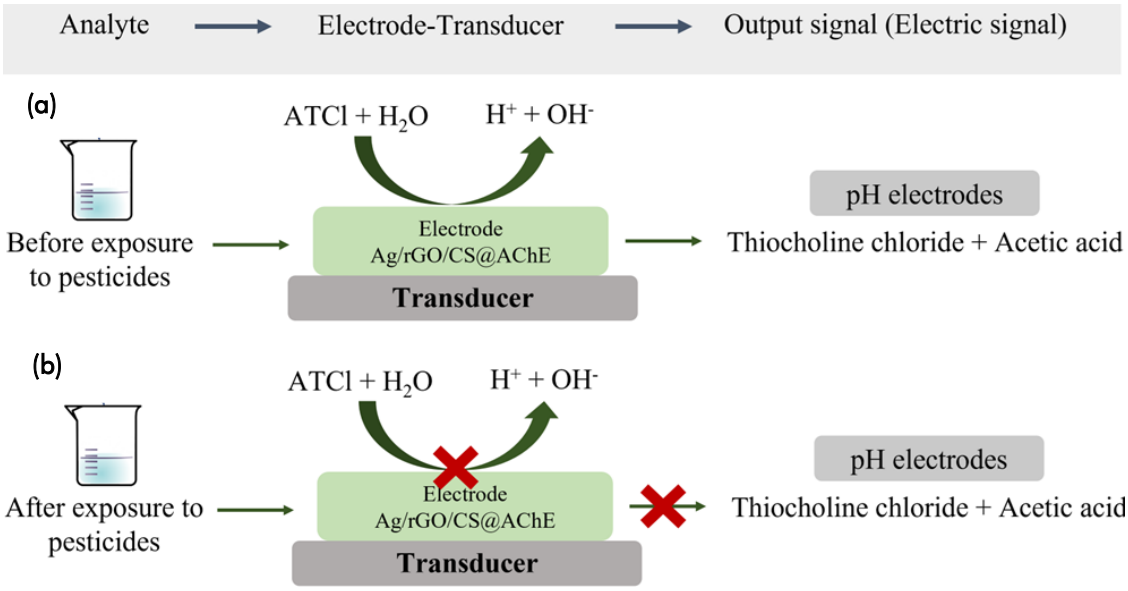
Figure 2(**c**), SEM-mapping, illustrates that the formed membrane has a gap between particles with a high density, indicating a large surface area of the membrane formed. Based on the morphology of the synthesized membrane, the research reported by An et al., (2018) indicates that the morphology of Ag/rGO/CS colloids is very dense and has a large surface area. The large surface area and contact area of the synthesized membrane affect the bonding power between the matrix and filler and cause an increase in the contact between the transducer and the enzyme as a biological element, resulting in effective adsorption of the target molecule. Naghib et al., (2018) reported that the large surface area of a membrane would increase the contact area between the composite and the target molecule, and the large contact area will significantly increase electron transfer. The atomic percentage of Ag/rGO/CS membrane using EDX (**Figure 2**(**d**)) showed elements O of 50.89% and C of 52.09% and Ag elements were successfully adsorbed on the surface of rGO with an atomic percentage of 8.25%. The CS coating was confirmed by N elements with a weight of 38.69%.

Optimization of the biosensor

Electrochemical biosensors are devices made with biological molecules (AChE enzymes) and electrodes. Functional enzymes are immobilized on the surface of the Ag/rGO/CS electrode to affect the selective determination of the analyte in the sample solution (Badawy & El-Aswad, 2014). **Scheme 2**, schematically the concept and structure of the electrochemical/potentiometer biosensor, where the surface of the Au electrode is modified with Ag/rGO/CS biomaterial, which can bind selectively to target analytes in sample solutions contaminated with many interfering substances, such as pesticides. Among the biosensors developed so far, enzyme-based biosensors have been studied the most because of their high sensitivity and wide application in environmental analysis, especially pesticides. At the surface of the electrodes, enzymes speed up chemical reactions. The products of these reactions are then oxidized or reduced by electrochemistry, creating an electric current as the output signal. Therefore, selectively the chemical signal (i.e., the type of analyte and its concentration) is transformed into an electrical signal through the enzyme/electrode interface (Anzai, 2015; Soropogui et al., 2018).

This biosensor relies on the potentiometric determination of the pesticide diazinon. In this study, the ATCl substrate was used for the AChE enzyme. AChE is an enzyme that helps break down acetylthiocoline (ATCh) or ATCl into thiocholine (TCh) or thiocholine chloride (TChCl) and acetic acid. The ATCl substrate is electrochemically active. Therefore, detectable via anodic oxidation at mV vs. Ag/AgCl, as shown in Scheme (Dimcheva & Horozova, 2015). The catalytic reaction is in **Figure 3** (Wei & Wang, 2015).

The above reaction occurs without an inhibitor. However, the addition of the organophosphate pesticide diazinon caused a significant decrease in enzyme activity. As a result, the formation of TChCl products will be reduced or even absent. The reaction on the biosensor was based on the inhibition of AChE by organophosphates (**Figure 3**) (Cesarino et al., 2012).



Scheme 2. Measure with the potentiometric method based on changes in pH (**a**) before and (**b**) after exposure to the pesticide.

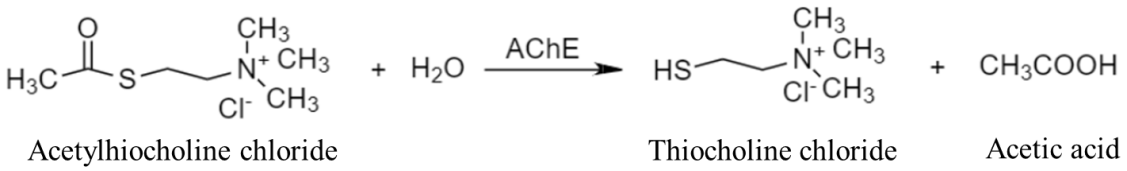


Figure 3. AChE enzyme reactions in ATCl substrate-based electrochemical sensors

Little or no TChCl causes a consequent decrease in the oxidation current measured for measuring pesticides (Yan et al., 2013). The problem is that the oxidation of interfering electroactive species takes higher potential, and thus TChCl's oxidation peak may be more significant, leading to relatively poor sensitivity (Wei & Wang, 2015; Yan et al., 2013). Using Ag/rGO/CS nanocomposite materials effectively reduces the possible need for TChCl oxidation since they have effective electron transport properties (Yan et al., 2013; G. Zhao et al., 2015). As the pesticide concentration rises, changes in the potential value of fluctuations in concentration lead to a decline in potential value (**Figure 4a**). This difficulty is due to the TChCl catalytic product's non-electrochemical nature. Therefore, the measurement of pH changes due to acid formation for evaluation of inhibition level, as shown in **Scheme 2**. Potentiometric indicators with pH-sensitive Ag/rGO/CS electrodes are commonly involved in this measurement.

The linearity value obtained from the curve of the potential value (mV) versus the -log pesticide concentration (**Figure 4**(**a**)) is used to determine sensitivity (Nernst factor) and measurement range. The working concentration range was analyzed to obtain the lowest and highest concentrations of natural pesticides as inhibitors. At **Table 1**, the 1:1:2 membrane's correlation value (R^2) is 0.9803, the 2:1:3 membrane's is 0.9836, and the 3:1:4 membrane's is 0.9850 in the measurement range of 10^{-8} to 1 μg L^{-1}. Measuring in buffer solutions has been linked to a linear relationship (potential vs. -log concentration) in other Nernstian systems. The sensitivity values of each membrane are 5.4667, 6.475, and 7.8133 mV/decade, respectively. In fact, according to Lai et al., (2017) and Özbek et al., (2021), the calibration curve's shape will depend on the ratio between buffer capacity and the amount of base (or acid) produced by an enzymatic reaction, being linear at low concentrations, Nernstian at high concentrations, and the type of mixture at medium concentrations (1×10^{-5} - 10^{-3} μg L^{-1} in this research). The highest sensitivity value was obtained at a 3:1:4 membrane, possibly due to the dominant Ag composition, thereby increasing the electron transfer value (Naghib et al., 2018). The pH-dependent surface charge of the conductive material can explain the low sensitivity of this sensor. In addition, the low sensitivity is also affected due to the low concentration of the test solution (Özbek et al., 2021). The difference in potential values did not cause a significant difference for all variations of the Ag/rGO/CS membrane composition.

Inhibition is one of the most important factors in pesticide analysis. **Figure 4**(**b**) shows that each pesticide inhibited AChE more effectively as the diazinon pesticide concentration increased. The presence of pesticide inhibitors will affect the performance of the enzyme in hydrolyzing ATCl into TChCl and acetic acid so that the phosphate group on the inhibitor will bind to the active site of the enzyme, namely the serine amino acid (Akyüz & Koca, 2019; Barkauskas et al., 2019; Loguercio et al., 2021). Thus, the product of the hydrolysis reaction will be reduced or not formed, which indicates a decrease in potential value. **Figure 4**(**b**), shows a diagram of the percentage of inhibition on pesticide concentration. From the three membrane compositions, the 3:1:4 membrane obtained the highest percentage of inhibition. Because it is related to the magnitude of electron transfer caused by the dominant Ag composition, the three Ag/rGO/CS membranes are suitable biosensor electrodes, can immobilized AChE enzymes, and are classified as good at detecting enzyme inhibition by pesticides. It was shown that the biosensor was 38.4504% inhibited by the pesticide diazinon.

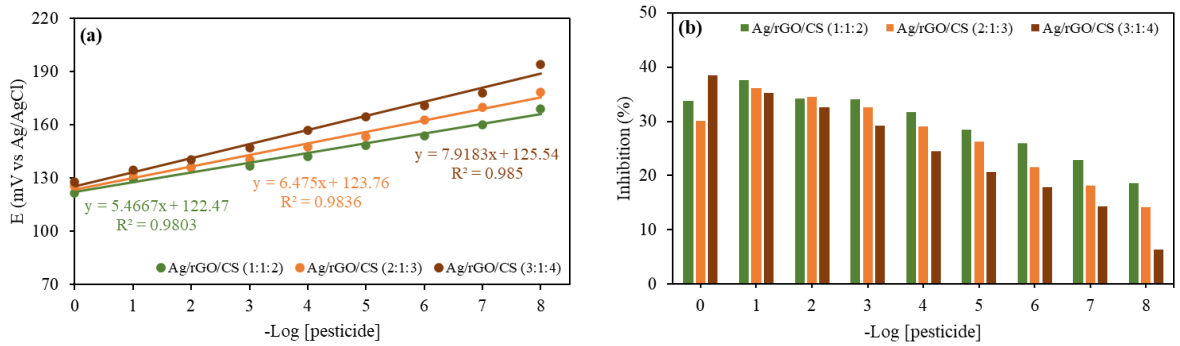


Figure 4. The plot of (**a**) E (mV vs Ag/AgCl) versus -Log [pesticide] and (**b**) inhibition (%) versus -Log [pesticide]

Table 1. Parameters of Biosensor

Composition of membrane Ag/rGO/CS	Parameters of Biosensor					
	Sensitivity	R^2	Range (μg L^{-1})	LOD (μg L^{-1})	SD	RSD (%)
1 : 1 : 2	5.4667	0.9803			0.0073	0.6870
2 : 1 : 3	6.4750	0.9836	1×10^{-8} - 1	1×10^{-7}	0.0334	1.2956
3 : 1 : 4	7.9183	0.9850			0.0356	1.2886

* rGO = reduced graphene oxide, CS = Chitosan, R^2 = Correlation value, LOD = Limit of Detection, SD = Standard of Deviation, RSD = Relative Standard Deviation

The LOD of the Ag/rGO/CS membrane electrode biosensor was 1×10^{-7} μg L^{-1}. The LOD of this work is well below the 50–100 μg L^{-1} of the Chinese National Standard (GB13192-91), as measured by Gas Chromatography (GC). The prepared biosensor exhibits a wide linear range and lower detection. **Table 1**, shows that the RSD values for each Ag/rGO/CS membrane are lower than 5%, which shows that the proposed biosensor is a promising biosensor for the practical detection of organophosphate group diazinon pesticides. According to (Christian, 2014), a good RSD value is < 5% for a 95% confidence level. The method is classified as suitable based on the RSD value obtained (Rahmani et al., 2018).

CONCLUSIONS

In this study, we have successfully constructed one type of potentiometric AChE biosensor based on Ag/rGO/CS with GTA as a crosslinking agent for diazinon determination. The proposed biosensor system is sensitive to small pesticide concentrations and is stable over time with repeated measurements, so it is declared to have good reproducibility and stability. In future research, nanomaterials such as metal nanoparticles and Au wires will modify rGO and CS. This material has good conductivity and can fill the gaps between rGO nanofragments, increasing the conductivity and improving the sensitivity of such sensors. It is hoped that this biosensor has potential applications in the simple, rapid, and quantitative detection of organophosphates.

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