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SPECTROSCOPIC ANALYSIS OF CHOLESTEROL ENZYME CHOX IN PVA ON THE INDICATOR ELECTRODE MEMBRANE OF CHOLESTEROL ANALYTE WITH THE POTENTIOMETRIC BIOSENSOR METHOD

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ABSTRACT

The spectrum pattern analysis of the urease enzyme immobilization technique in PVA, a PVA polymer that is soluble in warm water, has been carried out. There are differences in the spectrum patterns of PVA, PVA-KTpClPB and PVA-Enzyme-KTpClPB solutions, respectively UVA, UVB and UVC. The purpose of this study was to create a cholesterol sensor whose analyte cholesterol on the indicator electrode was coated with a PVA-Enzyme cholesterol (ChOx)/GA 2.9%/PANI-p-toluenesulfonic acid/PVC-KTpClPB-o-NPOE 61% membrane. The method used is the potentiometric biosensor method of ChOx enzyme immobilization technique in PVA, which is symbolized by (PVA-E ChOx). This study was conducted by comparing the indicator electrode membrane coated with PVA- variation of Enzyme cholesterol (ChOx)/GA 2.9%/PANI-benzenesulfonic acid/PVC-KTpClPB-o-NPOE 61%. ChOx enzyme was varied at 1:1 and 1:3. The results of the UV-Vis analysis of ChOx enzyme immobilization in PVA obtained a spectrum pattern that was the same as the ChOx spectrum pattern on the ChOx enzyme 1:1. After obtaining the best results from the PVA-E ChOx membrane at 1:1. Continued coating of the first indicator electrode GA 2.9% / PANI-p-toluenesulfonic acid / PVC-KTpClPB-o-NPOE 61%. The second electrode GA 2.9% / PANI-benzenesulfonic acid / PVC-KTpClPB-o-NPOE 61%. Both membranes were analyzed by EDX, XRD, and FTIR, each placed on a tungsten wire and glass preparation, to determine the best membrane. The best membrane is PVA-E1 ChOx / GA 2.9% / PANI-p-toluenesulfonic acid / PVC-KTpClPB-o-NPOE 61%.

Keywords: Spectrum Pattern; UV-vis; ChOx Enzyme in PVA; Membrane; EDX, XRD, and FTIR.

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INTRODUCTION

A study has been conducted on the manufacture of indicator electrodes with four-layer membranes, namely PVA-E/GA 2.9%/PANI-p-toluenesulfonic acid/PVC-KTpClPB-o-NPOE 61%.[1] The number of drops of urease enzyme was varied in a solution of 0.0350 g PVA and 10 mL of warm water. Urease enzyme 6 mg was dissolved in 0.5 mL (50% water: 50% alcohol). After mixing PVA with the enzyme, the variation in the number of drops was one drop and three drops, denoted by PVA-E one drop and PVA-E three drops. Previously, PVA, PVA-KTpClPB, and PVA-Enzyme-KTpClPB solutions were made. Looking at the spectrum pattern analyzed by UV-Vis, there were three absorbance spectrum patterns located at different wavelengths: Fig.-1(a), Fig.-1(b), and Fig.-1(c). Variations in the number of drops of urease enzyme showed a difference in the height of the absorbance spectrum pattern against the wavelength.[2] For the cholesterol enzyme ChOx, no variation in the number of drops was carried out because it only affected the absorbance height of the absorbance spectrum pattern against wavelength. Based on the change in the mixture in PVA affecting the absorbance spectrum pattern against wavelength, the active substance (KTpClPB) and plaster (o-NPOE) coating was carried out on PVC because PVC is insoluble in water (waterproof). GA coating creates cross-links between PVA-Enzyme and PANI conductive polymers so that electron transfer occurs. There are three polymers used, namely, PVA, PANI, and PVC. XRD characterization of sample A and sample B Fig.-2 and FTIR Fig.-3 have been carried out. Sample A is a

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PVA membrane-urease enzyme/GA 2.9%/PANI-p-toluenesulfonic acid/PVC-KTpClPB-o-NPOE 61%. Sample B is a PVA membrane-urease enzyme/GA 2.9%/PANI-p-toluenesulfonic acid/PVC-KTpClPB-o-NPOE 61%.[3] Based on this idea, a four-layer membrane[4] was made in this study by replacing the urease enzyme with the cholesterol enzyme (ChOx) in PVA as an enzyme immobilization technique with variations of the cholesterol enzyme 1:1 and 1:3, respectively, notations PVA-E1 ChOx and PVA-E2 ChOx. The best UV-vis analysis results were on PVA-E1 ChOx. To fulfill the potentiometric biosensor method, the PVA-ChOx Enzyme membrane was coated with GA 2.9%/PANI-p-toluenesulfonic acid/PVC-KTpClPB-o-NPOE 61% and compared with the coating of GA 2.9%/PANI-benzenesulfonic acid/PVC-KTpClPB-o-NPOE 61%. The aim is to obtain the best functional groups and FTIR spectrum patterns[5] transmittance to wave numbers.

EXPERIMENTAL

Material and Methods

The materials ordered at Sigma Aldrich are cholesterol oxidase enzymes from microorganism C8868 100UN. Glutaraldehyde (GA), PVC, KTpClPB, o-NPOE, Tetrahydrofuran; Polyaniline (PANI); Benzenesulfonic acid; p-toluenesulfonic acid, glass and tungsten preparations with a diameter of 1.0 mm. The method used is the potentiometric biosensor method[4–6] with variations in membrane coating, namely two layers, three layers, and four layers with p-toluenesulfonic acid and benzenesulfonic acid solvents. The first layer is a PVA polymer layer, the second layer is glutaraldehyde, the third layer is a PANI conductive polymer, and the fourth layer is a PVC polymer. Immobilization techniques[6,7,8] cholesterol enzyme (ChOx) in PVA for cholesterol analytes. PANI is a conductive polymer, so electron transitions occur on the membrane. PVA is a place to immobilize cholesterol enzymes, and GA is a cross-link between PVA-Enzyme and conductive polymer. PANI conductive polymer depends on the conductive polymer solvent acid, there are two acids used, namely p-toluenesulfonic acid and benzenesulphonic acid. The best ChOx enzyme composition is 4.8 mg in 0.5 mL consisting of (50% water: 50% alcohol). All polymers are in a 1:1 ratio, namely 35 mg PVA, 35 mg PVC, and 35 mg PANI, while the active substance is 50 mg KTpClPB, plasticizer (o-NPOE) 61%.[9,10,5] The membrane is made of four layers (1) PVA-E1-ChOx/PVC-KTpClPB-o-NPOE 61% as sample ChOx1; (2) PVA-E1-ChOx/GA-2.9%/PVC-KTpClPB-o-NPOE 61%[2], sample ChOx2; (3) PVA-E1-ChOx/GA-2.9%/PANI-p-toluenesulfonic acid/PVC-KTpClPB-o-NPOE-61%[11], sample $ChOx_3$; (4) PVA-E1-ChOx/GA 2.9%/PANI-benzenesulfonic acid/PVC-KTpClPB-o-NPOE-61%, as sample ChOx4, Fig.-5 Edx analysis.

RESULTS AND DISCUSSION

UV-vis Analysis of Urease Enzyme Immobilization

Atomic absorption spectroscopy is an analytical chemical procedure that uses the principle of energy absorbed by atoms. Spectroscopy is divided into four groups, namely (a) absorption, (b) emission, (c) scattering, and (d) fluorescence. UV-vis spectrophotometer[12] is used to measure the absorbance of a liquid that has a chromophore group to a certain wavelength of light. Spectrometry is a spectroscopic method[13] that measures the amount of energy absorbed by matter with the criteria of ultraviolet (UV) light at a wavelength between 200 to 400 nm and visible light at a wavelength of 400 to 750 nm. UV-visible spectroscopy[14] is used to analyze the chemical properties of a material. UV-Vis spectrophotometry can detect compounds that have color or chromophore groups in a sample. In addition, it determines concentration, identifies unknown compounds, and provides information about the physical and electronic structure of organic and inorganic compounds. UV sensors energize electrons that cause an electric current. Electrical conduction is the transfer of electrically charged particles through a medium. The electric current will be stronger if it gets brighter light. Shimadzu 1800 UV-vis spectrophotometry is used to measure the absorbance of liquid samples using wavelengths in the UV region (200-350 nm) and visible light (350-900 nm). Ultraviolet light is in the 100-400nm wave band, which is divided into UV-A, UV-B[15], and UV-C[16], with details of UV-A between 315 - 400 nm, UV-B between 280 - 325 nm, and UV-C between 100 - 280 nm. UV-A is the longest wavelength group, UV-B is the medium wavelength group, and UV-C is the shortest wavelength group.

Absorbance is the power of light radiation absorbed by a solution, either a standard solution or a blank. A blank solution is a solution that does not contain an analyte. A blank solution is a sample that only contains

solvent. Absorbance is used in spectroscopy and analytical chemistry to determine the content and concentration of a substance. The concentration of the solution analyte is determined by measuring the absorbance at a certain wavelength according to the Lambert-Beer law.[17] Ultraviolet and visible spectrometry are useful in determining functional groups of organic compounds and quantitative analysis. Chromophores are molecules that absorb light strongly in the UV-Vis region, for example, hexane, acetone, acetylene, benzene, carbonyl, carbon dioxide, carbon monoxide, nitrogen gas. The purpose of determining the maximum wavelength is to determine the optimal absorbance value of a compound using UV-Vis spectrophotometry.

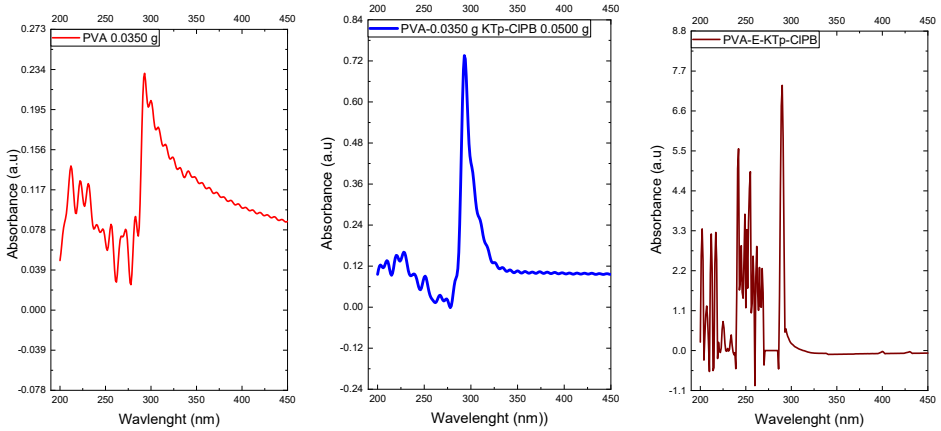

Fig.-1: Absorbance Spectrum Pattern Against Wavelength of at: a) 0.35 mg PVA, b) 0.35 mg PVA 0.50 mg KTpClPB, c) PVA-E-KTpClPB.

Analysis of the absorbance spectrum pattern against the wavelength determines the UV-A, UV-B, and UV-C classes; the relationship of these wavelengths reflects the greatest absorbance energy. The greatest absorbance energy is located at a short wavelength, according to Fig.-1(a) the type of UV-A, 1(b) UV-B and 1(c) UV-C thus the best UV-Vis spectrum pattern on the membrane containing the PVA-E-KTpClPB mixture. Based on the idea of the mixture used in the study, an indicator electrode membrane was designed with a layer of sample ChOx3 and sample ChOx4. Sample ChOx3 and sample ChOx4 were analyzed by UV-Vis first Fig.-4. The following membrane on the indicator electrode of sample ChOx3 and sample ChOx4 was analyzed by FTIR Fig.-5. The preparation of sample C and sample D was based on sample A and sample B, consisting of sensor elements where the urea analyte was replaced with cholesterol analyte. After UV-Vis analysis was carried out, XRD analysis was continued for sample A and sample B. Figure-2 was also analyzed by FTIR, Fig.-3, seeing the relationship between the two-layer membrane and the four-layer membrane used for sample ChOx3 and sample ChOx4.

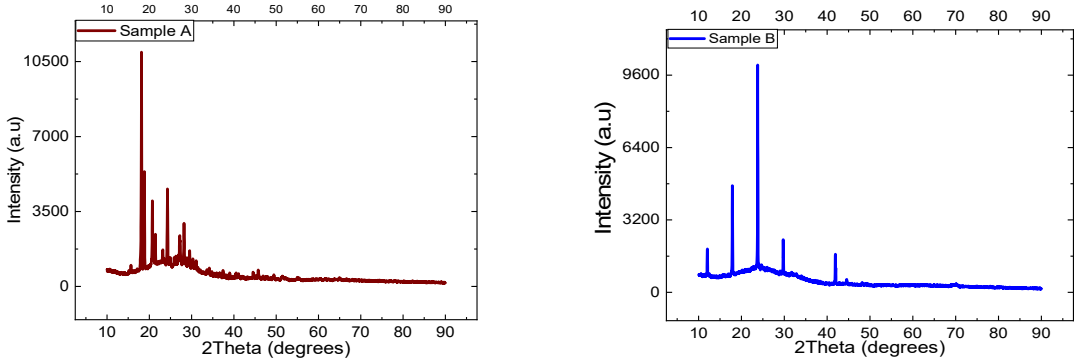

Fig.-2: XRD Diffraction Spectrum Pattern Against Diffraction Angle 2theta of Sample A and Sample B

XRD Analysis of Urease, p-Toluenesulfonic Acid and Benzenesulfonic Acid Membranes

The analytical method used to identify the crystal phase in the material by determining the parameters of the lattice structure and obtaining the particle size is X-ray Diffraction (XRD). The peak width is inversely

proportional to the crystal size. Thinner peaks indicate larger crystals. Wider peaks indicate smaller crystals, defects in the crystal structure, or that the sample is amorphous. The crystal spectrum pattern of the results of this study still has an amorphous spectrum pattern, a solid that does not have perfect crystallinity. Modification of the membrane layer[18] for the indicator electrode is based on the biosensor potentiometric method. Biosensors are devices that measure enzymatic reactions by producing signals that are proportional to the concentration of the analyte. Biosensors consist of biological receptors or sensor elements and transducers. The sensor element is the enzyme cholesterol ChOx in PVA with enzyme immobilization techniques. The best membrane from this study has four layers, with the first layer being PVA-Enzyme ChOx, the second layer being GA-2.9%, the third layer being PANI-p-toluenesulfonic acid sample A and PANI-benzenesulfonic acid sample B, and the fourth layer being PVC-KTpClPB-o-NPOE 61%. The XRD spectrum analysis pattern is shown in Fig.-2 for the sample membrane (A) ChOx3; (B) ChOx4. There is a difference in energy intensity in sample A than in sample B. The XRD spectrum pattern at the highest intensity in Fig.-2, according to the analysis of the EDX spectrum pattern from previous studies where the highest voltage range is located at the tungsten atom (wolfram) as the working point of the tungsten indicator electrode. There is a difference in the high energy intensity between sample A and sample B, as well as the location of the 2theta diffraction angle with different membrane materials, namely in the third layer of sample A PANI-p-toluenesulfonic acid and sample B PANI-benzenesulfonic acid. An acid is a substance that can produce hydrogen ions (H^+) when dissolved in water. Acids are substances in water that can ionize so that they release hydrogen ions (H^+) or hydronium ions. A base is a substance in water that can ionize so that it releases hydroxide ions (OH^-). An acid is a substance that can donate protons in its reaction. The base is a substance that can accept protons in its reaction, while protons in chemical reactions are identical to electrons. Ionization is a process in which atoms or molecules acquire a negative or positive charge by gaining or losing electrons. If an atom or molecule gains electrons, it will be negatively charged (anion)[19], and if it loses electrons, it will be positively charged (cation). The indicator electrode functions as a cation so that it is suitable for use as a solvent for conductive polymers of p-toluenesulfonic acid or benzenesulfonic acid, each at a concentration of 2 M of 0.35 mg PANI. The cathode attracts positively charged cations. The anode attracts negatively charged anions.[20]

Table-1: Table of Intensity Against Diffraction Angle 2Theta of Sample A and Sample B

2Theta (degrees)	Intensity (a.u) Sample A	2Theta (degrees)	Intensity (a.u) Sample B
18.08542	4941	23.75771	4426
18.11168	6974	23.78397	8450
18.13794	9618	23.81023	10048
18.16421	10940	23.83649	6639
18.19047	9451	23.86275	5810
18.21673	6289	23.88901	3297

According to Table-1, from sample A and sample B, there is a difference in the diffraction angle of sample A of around 18 degrees with the highest intensity of 10940 sample B of around 23 degrees with the highest intensity of 10048. The difference is due to p-tolinesulfonic acid and benzenesulfonic acid PANI solvents. According to[21], the acidity level is very influential between HCl and H_2SO_4, as well as between p-toluenesulfonic acid or benzenesulfonic acid, as seen in Fig.-2.

FTIR Analysis of Urease Membrane, p-toluenesulfonic Acid and Benzenesulfonic Acid

FTIR spectroscopy is one of the analytical techniques used to identify organic materials, polymers, and inorganic materials. FTIR spectroscopy is one of the analytical methods based on the principle of chemical compound interactions that produce vibrations of polyatomic chemical bonds or functional groups of chemical compounds. Transmittance (T) is the ability of a material to transmit light or radiation, referring to the amount of light energy absorbed, scattered, or reflected. The relationship between concentration and absorbance is directly proportional, the greater the concentration, the greater the absorbance, and conversely inversely proportional to transmittance, the smaller it is. Absorbance provides important information about the electronic structure of molecules. Electron structure is the condition of electron movement. Electrons play an important role in electricity, magnetism, chemistry, and thermal conductivity. This means that the

greater the transmittance value, the greater the transfer of electrons from one atom to another or molecule to another molecule. According to Fig.-3, sample A has a much larger %Transmittance than sample B, with sample A having the third layer of PANI-p-toluenesulfonic acid and sample B PANI benzenesulfonic acid. Each comes from the membrane sample membrane (A) ChOx3; (B) ChOx4. P-toluenesulfonic acid is an aromatic sulfonic acid that has strong acidic properties and is used as an acid catalyst, soluble in water, alcohol, and other polar organic solvents. P-toluenesulfonic acid is a strong organic acid, one million times stronger than benzoic acid, one of the strong acids in solid form.[22] In addition, unlike other strong mineral acids (especially nitric acid, sulfuric acid, and perchloric acid). Benzenesulfonic Acid is a strong acid that has very acidic properties. Used in various industrial processes, such as detergent production, sulfonation of aromatic compounds, and as an intermediate in the manufacture of medicines and dyes.

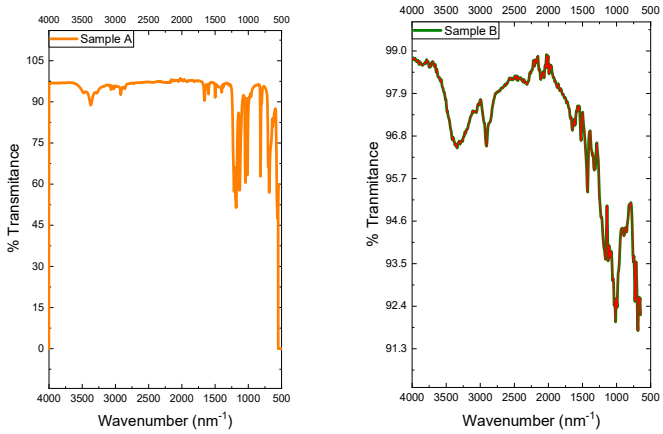

Fig.-3: FTIR Transmittance % Spectrum Pattern Against Wave Number of (a) Sample A and (b) Sample B

UV-vis Analysis of ChOx Cholesterol Enzyme Immobilization

Reasoning from absorbance is the radiation power of light absorbed by the solution, both standard solution and blank by sample A and sample B. So, sample C and sample D no longer use blanks directly in the standard solution. According to[18], PVA-E ChOx 1:1 sample C and PVA-E ChOx 1:3 sample D with the number of drops of ChOx enzyme on PVA is three drops. The absorbance spectrum pattern can be seen in Fig.-4(a) and Fig.-4(b) analyzed with the cholesterol spectrum pattern in Fig.-4(c). The cholesterol spectrum pattern is similar to the absorbance spectrum pattern in Fig.-4(a), namely PVA-E ChOx 1:1.[23]

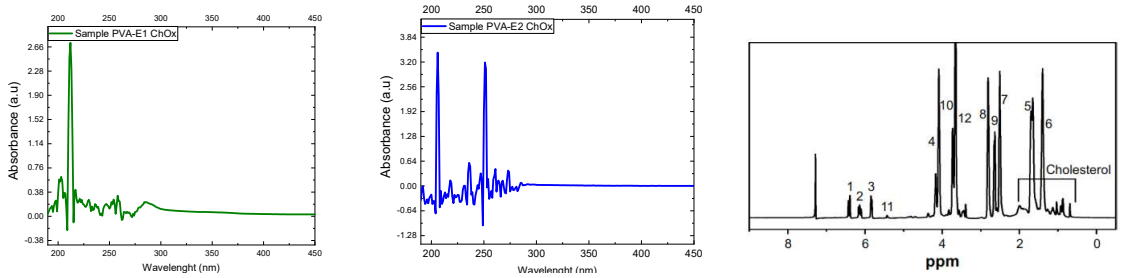

Fig.-4: UV-Vis Absorbance Spectrum Pattern Against Wavelength of (a) Sample ChOx3, (b) Sample ChOx4, and (c) Cholesterol Spectrum Pattern[23]

XRD Analysis of ChOx Cholesterol Enzyme Immobilization on Several Membrane Variations

Immobilization analysis of ChOx cholesterol enzyme only for PVA-E1-Chox-PVC-KTpCIPb-o-NPOE-61%, denoted ChOx1 see Fig.-4a; PVA-E1-ChOx/GA-2.9%/PANI-benzene-sulfonate-PVC-KTpCIPb-o-NPOE-61%, denoted ChOx4 see Fig.-4b. Studying the variation of two-layer membrane with four layers, it turns out that there are differences in the XRD spectrum pattern from the intensity (a.u) to the 2theta diffraction angle. The height of the intensity peak depends on the membrane layer and its contribution, the second PVC membrane see Fig.-4a. Polyvinyl chloride is a thermoplastic polymer material. Polymers are repeating chains of long atoms formed from binders in the form of identical molecules called monomers.

Its properties depend on the added plasticizer. The form of the level of rigidity (flexibility) is used as insulation, so that it is not isolated, the active substance KTpCIPb is added. A plasticizer is a plastic additive that functions to reduce polymer stiffness so that an elastic and flexible layer is obtained. This sensor in the fourth layer contains o-NPOE as a plasticizer and KTpClPB as an ionic additive to the PVC matrix.[24] To increase the ability of the PVC membrane, it is necessary to add a plasticizer nitrophenyloctylether (NPOE), a counter anion of potassium tetrakis-chlorophenylborate (KTpCPB). Glutaraldehyde (GA) compound $C_5H_8O_2$ contains two aldehyde groups and is used as a biological tissue binder. Reacts with primary amine and thiol groups, which are functional in proteins, nucleic acids, and polymer materials. Conductive Polymers are organic polymers that conduct electricity (electron transfer). The conductive polymer material used in this study is PANI. PANI can dissolve in 2 M p-tolunesulfonic acid and 2 M benzenesulfonic acid

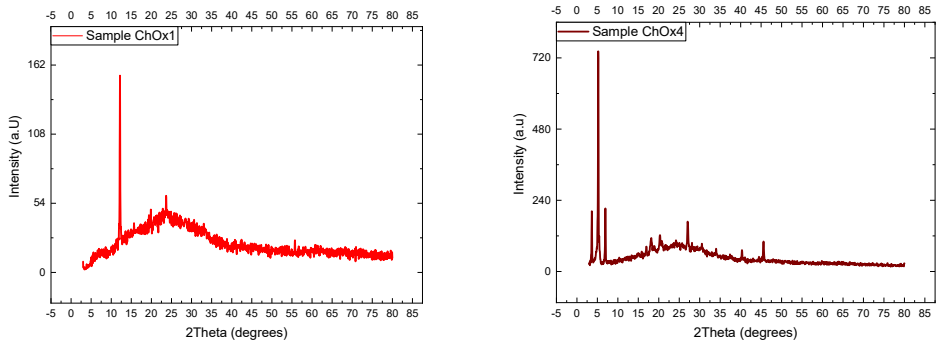

Fig.-5: XRD Diffraction Spectrum Pattern Against 2theta Angle of (1) Sample ChOx1 (2) Sample ChOx4

EDX Analysis of Indicator Electrode Membrane

Figure-6 EDX analysis of electrode membranes containing conductive polymers (a) sample ChOx1, (b) sample ChOx2, (c) sample ChOx3 (d) sample ChOx4, respectively. The EDX spectrum pattern of intensity to energy shows an increase in intensity due to the addition of 2.9% GA as a cross-link between PVA and PVC containing the active substance KTpClPB, and the spectrum pattern is similar. Likewise, figure 6 (d) contains 2 M benzenesulfonate PANI, but the intensity decreases with the number of layers four. While Fig.-6 (c) only differs in the acid used, namely 2 M p-toluenesulfonic acid, there is a change in the spectrum pattern with a smaller intensity. Of course, this difference is caused by the p-toluenesulfonic acid and benzenesulfonic acid in PANI. The electron transfer of p-toluenesulfonic acid[25,26] is greater than that of benzenesulfonic acid. Electron transfer[27] is the movement of electrons in redox reactions and ionic bonds, with oxidation involving the loss of electrons. Electron transfer involves changes in the oxidation state of the reactants and their products. It is natural that in the four-layer membrane, the intensity of its atomic units decreases against its energy from this EDX analysis because the nature of p-toluenesulfonic acid is an alkali, while benzenesulfonic acid is a phenol.

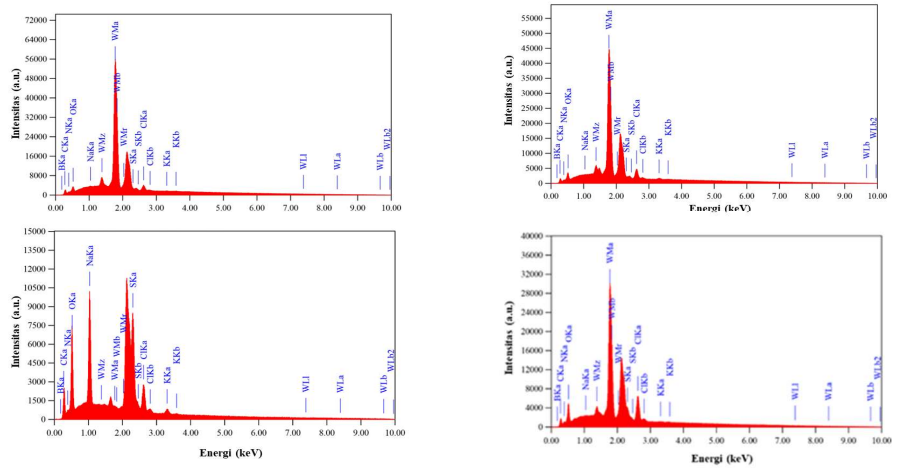

Fig.-6: EDX Spectrum Pattern of Intensity Versus Energy of (a) Sample ChOx1, (b) Sample ChOx2, (c) Sample ChOx3, (d) Sample ChOx4.

FTIR Analysis of Indicator Electrode Membrane

P-Toluenesulfonic acid is used in esterification reactions[28], acetalization, etherification, and alkylation. Esterification is a direct reaction between carboxylic acids and alcohols. Toluenesulfonic acid is an aromatic sulfonic acid[20] used as a catalyst[29] strong acid.[30] Aromatic compounds are chemical compounds consisting of a conjugated planar ring system accompanied by a delocalized pi electron cloud. The ring structure[22] is planar, meaning that all atoms in the molecule lie in the same plane. The conjugation system occurs in organic compounds whose atoms are covalently bonded singly and double alternately (C=C-C=C-C) and influence each other to form an electron delocalization area.[31] Delocalization is a condition in which electrons can move from one atom to another.

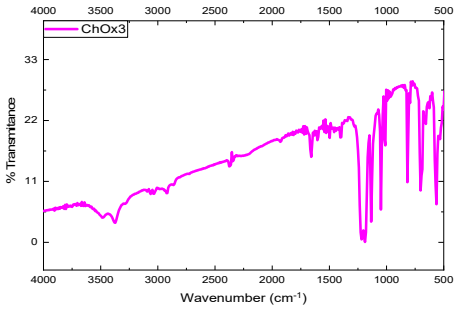

Fig.-7: Spectral Pattern of % FTIR Transmittance against Wave Number of $ChOx_3$ Sample

Esterification can be done with acid catalysts such as HCl and H_2SO_4. Acetylation is an organic esterification reaction using acetic acid in chemistry. Acetylation is a chemical reaction that replaces hydrogen groups with acetyl groups (CH_3CO) in a compound. Etherification is a reaction to make ethers based on a substitution reaction. Alkylation is a chemical reaction of adding an alkyl group by a substitution or addition process[32] to an organic compound. Benzenesulfonic acid (benzenesulfonic conjugate base) is an organosulfur compound with the formula $C_6H_6O_3S$. Benzenesulfonic acid is useful for producing phenol by combining it with sodium hydroxide or hydrolyzing one of its salts, usually sodium. Phenol compounds are organic compounds that have a hydroxyl group bound to a benzene ring. Phenol is similar to alcohol but forms stronger hydrogen bonds; this compound is more soluble in water[33] than alcohol. The spectrum pattern of urease enzyme in Fig.-3(a) is very different from the spectrum pattern of cholesterol enzyme seen in Fig.-7, while the spectrum pattern of PANI p-toluenesulfonate acid remains similar, namely the wave number range of 400 - 1500 cm^{-1}. The difference in the spectrum pattern lies in the % transmittance to wave numbers between 1500 - 4000 cm^{-1}, which consists of the ChOx1 sample layer. The prediction of Fig.-3(b) has the same properties as the immobilization of the urease enzyme from PANI-benzenesulfonate. So that the cultured sample is obtained in the $ChOx_3$ sample. This spectrum pattern is by the spectrum pattern according to.[34]

CONCLUSION

The analysis in this study is the analysis of the variation of the indicator electrode membrane whose cholesterol analyte comes from the cholesterol enzyme ChOx, the best results in the ChOx3 sample.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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