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Literature Review

Immunosensor fabrication methods: a scoping review

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Abstract
Various methods of establishing a diagnosis, especially for initial diagnosis, have been widely developed, such as Rapid Diagnostic Test (RDT), Enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR). However, those tools have several different limitations in each tool. The limitations of this tool include a fairly high error rate and the need for complex tools. The work can only be used by special personnel and a long processing time. Immunosensors are tools that can be used as an alternative. Immunosensor is a sensor used to detect specific immune reactions between antibodies and targets in the form of antigens and can detect interactions between analyte targets and antibodies from changes in electrochemical signal, sot the examination time is relatively faster. This scoping review aims to review each immunosensor fabrication parameter. The results proved that each analyte has a different characterization and is very diverse. So it is necessary to select the right parameters (electrode type, immunoassay configuration, electrode modification, receptor immobilization, and electrochemical characterization). The linear range and detection limit are also important parameters that can be developed so that very limited analyte concentrations in the sample can be detected. It is necessary to review fabrication methods to improve the stability of immunosensors so that the ligands contained in the immunosensor electrodes can last a long time to be able to carry out mass production.

Keywords: electrochemical characterization; electrode modification; immunosensor; linear range; limit of detection

1. Introduction

Various methods of establishing a diagnosis, especially for initial diagnosis, have been commonly developed, such as Rapid Diagnostic Test (RDT), Enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR). However, these various tools have several different limitations in each tool. The limitation of this RDT tool is in the test results. In this tool, the test results are qualitative, so the results obtained cannot be analyzed further and have a fairly high error rate (Chevaliez &; Pawlotsky, 2018). In the ELISA Method, the limitation is that the testing time is quite long, where this tool takes ± 3 hours and requires complex special tools and instruments in a laboratory environment. At the same time, the process can only be done by officers with special expertise (Tyagi et al., 2020). Another tool, namely PCR, is also similar. The limitations of this tool include the need for a more sterile space to avoid contamination, high costs, and requires a long time (2-5 hours) depending on the type of test instrument and reagent used (Teymouri et al., 2021). Therefore, there is a need for alternative tools that are more effective in terms of the equipment used, officer capabilities, the accuracy of results, and length of processing time.

Immunosensor is a detection device or element capable of quantifying the interaction between antibody and antigen bonds (Aydin et al., 2021). Transducers detect and convert specific interactions between antibodies and antigens into electrical signals (Moina &; Ybarra, 2012). The amount of electrical signal produced by the transducer depends on the concentration of the analyte. This biosensor was first developed in 1962 by Clark and Lyons to determine the oxidation state of glucose solutions

(Clark &; Lyons, 1962). The use of biosensors based on enzyme immobilization was first made by Guilbault in 1963 to detect nerve agents in the atmosphere. Then it developed until the 1990s. Adam Heller explained the concept of binding directly to receptors such as Enzymes, Antibodies, Antigens, DNA, Proteins, and other biological agents to electrodes and converting analytes into products that can be quantified using transducers (usually electrochemical, optical, or piezoelectric Devais). Adam Heller's concept is still used today. Based on the combination of antigen-antibody recognition and high sensitivity in electrochemical methods, an Electrochemical Immunosensor (EI) has a reliable and significant ability to perform early diagnosis and clinical analysis in the realm of disease, food safety control, environmental monitoring, home health care, and so on (Ranjan et al., 2021).

Electrochemical Immunosensor is a type of biosensor that principally adsorbs antigen species or antibodies on signal transducers that will detect bonds between species (Polat et al., 2022). EI has high sensitivity, easy operation, and does not require special qualifications and skills during the testing process. In addition, testing and detection sites do not have to be carried out in laboratories with high safety standards and instruments with low costs. EI is prospective to be developed in Point of Care (POC) applications because of the compact instrument and sensor size, as well as the potential to be developed in automation-based systems where there is no need for significant human intervention to carry out the testing process (Bonini et al., 2021). EI is qualitatively able to detect biological analyte targets ranging from small molecules (such as hapten and natural toxins) and macromolecules (antigens and biomarkers in disease) to cells, pathogenic bacteria, or viruses (Polat et al., 2022).

Some reviews of immunosensor have been carried out frequently in recent years. However, the reviews specifically discuss specific steps and analytes only. The review includes the fabrication and application of immunosensors based on electroactive materials for the early detection of various cancers (Z. Li et al., 2022), fabrication of immunosensors for the detection of influenza virus pathogens (Zhang &; Miller, 2019), immunosensors for detecting drugs (Gandhi et al., 2015). This review aims to discover each immunosensor fabrication parameter, not only specific reviews of certain steps or analytes. Each analyte has different characteristics in producing immunosensors. So far, research on how immunosensors work as a diagnostic tool is still rarely carried out. This is the main reason that prompted this literature study to be carried out to provide alternative solutions in obtaining diagnostic enforcement tools that are more effective, efficient, and in a short time.

2. Research Methods

This sub-chapter will explain some steps of conducting a scoping review related to immunosensor fabrication, from literature search to results exposure. A literature search is carried out using keywords in the form of "Immunosensor", "Modification of electrode", "Immunoassay", "Printing electrode", "Biosensor", "Immobilization of electrode", "Diagnosis of tropical disease", "Characterization of electrochemical sensor". There are also other keywords specifically for finding immunosensor parameters, such as writing the type of analyte, for example "Immunosensor for cancer detection", "Bacteria Immunosensor" to "Immunosensor for tropical disease". Article searches are carried out in several databases: ScienceDirect, Multidisciplinary Digital Publishing Institute (MDPI), Springer, Royal Society of Chemistry (RSC), Pubmed, and google scholar.

These review results aim to systematically group the series of immunosensor fabrication ranging from electrodes, electrode modification, and immobilization of receptors and analytes to electrochemical transduction. If the literature does not have clear parameters for each step of its fabrication, then the author will make an exception to the literature. The author tries to make the primary literature as varied as possible by providing several examples of fabrication techniques and examples: of types of samples and/or analytes, including detection limits (LoD) and linear ranges. The literature used in this study includes several related criteria. First, the literature published within the last ten years

(2013-2023); Second, the articles discusses the steps of Immunosensor fabrication from the beginning to the formulation of results; Third, the literature used to identify sensor quality is the linear range and detection limit; Fourth, the literature must contain these results with the target of research institutions or health workers in the field of clinical pathology and other biomedical development actors.

The article selection process is carried out by looking at several components: Title, Title keyword, and keyword. The first marker in searching for literature with relevant titles and keywords will increase the chances of getting articles according to the needs of the literature to be used, whether it is in the primary or secondary category. The second is to read the abstract. A good abstract provides an overview of the entire research because the abstract generally contains objectives, methods, and results. The third step is creating a graphic abstract. Research on fabrication will generally load the entire fabrication series into a structured drawing for each step. The last is to conclude. The conclusion answers the objectives supported by the study's results. This will be enough to help find a summary of the research results.

3. Results and Discussion

The total number of articles found amounted to 672 based on search results in the database. The articles are sorted by title according to inclusion criteria. Two hundred and seventeen articles were indicated to be off-topic, so they were excluded, and 455 articles remained. Then these remaining articles are sorted again by abstract and abstract graphics. As a result, there were 288 articles issued because the publication time was more than 10 years. The last step is accessing the full-text article. One hundred ten articles were issued at this step because they did not clearly discuss the fabrication steps. The remaining 57 articles were used in this review. The following is the flow of the article selection process summarized in figure 1.

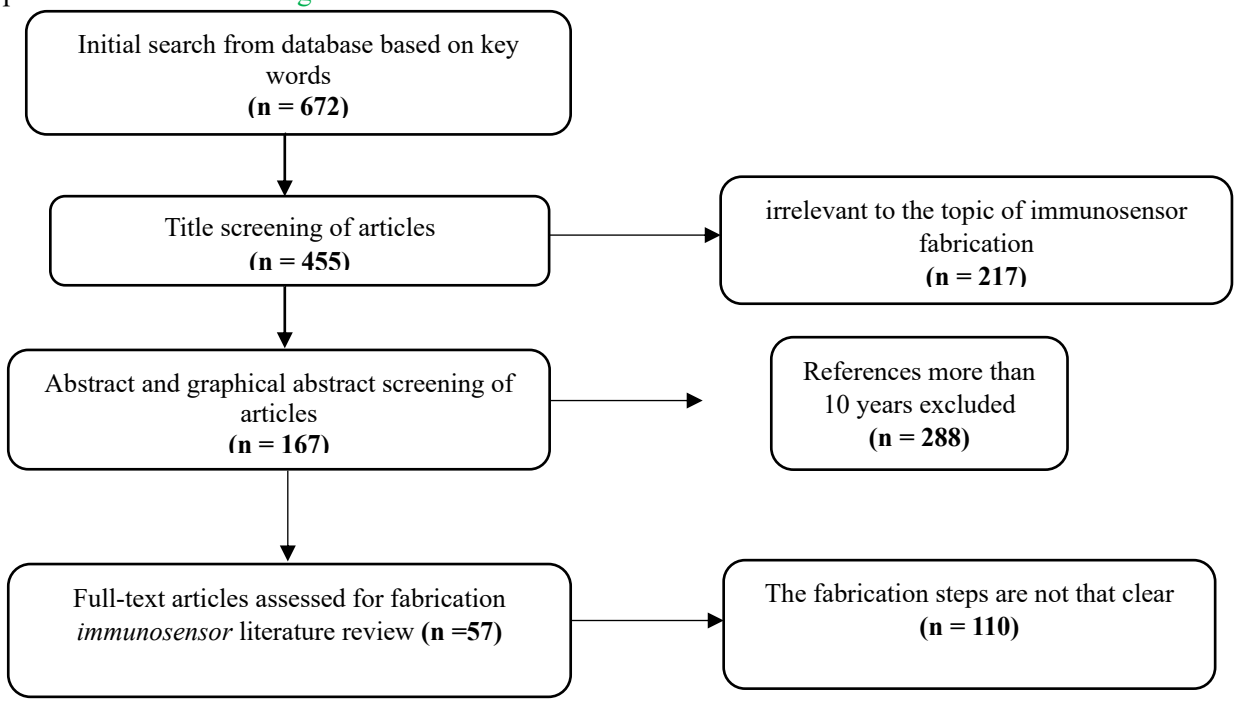

Figure 1. Article selection process

This review discusses some of the results of immunosensor research since the last 10 years, where each parameter will use different techniques depending on the type of sample or analyte to be detected and how much value will be optimized for the detection limit and linear range.

3.1.Selection of Sensor Electrode

The electrode is an important component in the sensor because it is the element that identifies the target to be detected directly and converts it into an electrical quantity or output in the form of an electrical signal. In EI sensors, there are generally three electrodes, namely Working Electrode (WE), Reference Electrode (RE), and Auxiliary Electrode (AE). The three electrodes have different functions. Working Electrode is the electrode where electrochemical reactions occur. The ideal working electrode is a WE that has a reproducible surface area and low background current. This Working Electrode consists of several types, namely mercury electrode (Dides et al., 2021), solid/rod electrode (Emir et al., 2021), modified electrode (Mat Zaid et al., 2020), and microelectrode (Russell et al., 2019).

Reference electrodes produce a constant potential during operation and are relatively stable to temperature changes. This can be used as a benchmark for potential changes in WE to measure the potential in cells or analytes. The auxiliary electrode acts as a place where electrons enter so that current can be passed through the cell, but this electrode does not affect the reaction at the working electrode. As a place of entry of electrons, the cross-sectional area of AE must be wider than WE. Generally, AE uses inert metal materials (Inezia Aurelia, 2005), while WE materials must significantly influence the possibilities and limitations of electrochemical methods. In the implementation of the oxidation process, less active metals are commonly used materials (Josypčuk et al., 2019).

The fabrication of an EI sensor usually occurs on electrodes that have been modified. The goal is to enrich the electrode material with electrons so that the current produced will be much greater than the electrode that is not modified. The results of this review will explain the correlation between electrode modifications in certain analytes and the results in the form of detection limits and linear ranges obtained. Electrode selection is the main thing in the immunosensor fabrication process. Screen Printed Electrode (SPE) is usually used for commercial-scale immunosensor fabrication because it is cheap, compact, and easy to mobilize. The condition of electrodes that have been mobilized with ligands or receptors makes the end user no longer need to fabricate the electrode modification process. Screen-printed electrodes are usually disposable electrodes for a single test.

In contrast, Glassy Carbon Electrode (GCE) is more commonly used on a research scale because it can be used repeatedly with certain cleaning treatments. While the Carbon Paste Electrode (CPE) requires the end user to carry out the Immunosensor fabrication process from the electrode manufacturing stage, so it is unsuitable for commercial purposes. Immunosensors based on the selection of electrode types are summarized in table 1.

Table 1. Types of immunosensor electrodes

Elektroda	Receptor	Analyte	Linear Range	LOD	Reference
Carbon black - **SPE**	Lacasse enzyme	Catechol	2.5–50 µ mol/L	2 µ mol/L	(Castrovilli et al., 2020)
SPCE/AuNP-Poly(amidoamine)	Anti–tau capture antibody (Cab)	Tau protein	–	1.7 pg/mL	(Razzino et al., 2020)
GCE/Pgo@aU	Anti-HBeAg	HBeAg	0.1 fg/mL–500 pg/mL	26 fg/mL	(Gao et al., 2018)
GCE/GO-CS	Anti-HBsAg	HBsAg	0.1–300 pg/mL	60 fg/mL	(N. Alizadeh et al., 2018)
IIP-**CPE**	Anti-CE 3+	CE 3+	1,5 x 10^{-7} mol/L	1 x 10^{-6}-1 x 10^{-5} mol/L	(T. Alizadeh et al., 2016)
IIP-**CPE**	Anti–CD 2 +	CD 2+	4–500 nM	1,94 nM	(Samandari et al., 2019)

Immunosensor to detect catechol in samples in the form of a buffer made on SPE with modifications in the form of black carbon, then Lacasse enzyme is mobilized on the electrodes. When electrochemical characterization was carried out, linear range results were obtained of 2.5-50 µ mol/L and a detection limit of 2 µ mol/L (Castrovilli et al., 2020). Razzino et al. (2020) developed immunosensors with SPE electrodes modified with carbon and nanoparticles in the form of gold with receptors in the form of Anti-Tau capture antibody (Cab) and flow targets in the form of Tau Protein for Alzheimer's diagnosis. The detection limit for Tau protein concentration reached 1.7 pg/mL. The research by Gao et al. (2018) explained the fabrication of immunosensors to detect the Hepatitis B virus protein (HBeAg). If the patient has HBeAg protein, then the patient is at risk for transmitting the Hepatitis B virus. The study used the Glassy Carbon Electrode (GCE), where Protein HbeAg was detected based on the concentration bound to the anti-HbeAg immobilized at the electrode. The results were quite good because the linear range obtained was 0.1 fg/mL-500 pg / mL, and the detection limit was 26 fg / mL.

Another study related to hepatitis was carried out by N. Alizadeh et al. (2018) with electrodes in the form of GCE modified with graphene oxide and chitosan to detect the presence of hepatitis antigens in serum extracted from the patient's blood. The analyte target is HbsAg, with a linear range result of 0.1-300 pg/mL and a detection limit of 60 fg/mL. The target analyte is HbsAg. The result is a linear range of 0.1-300 pg/mL and a 60 fg/mL detection limit. Meanwhile, Samandari et al. (2019) used CPE-based electrodes modified with Ion Imprinted Polymer for CD 2+ detection with receptors in the form of anti-CD 2+. The result is a linear range of 4-500 nM and a detection limit of 1.94 nM.

3.2. Effect of Electrode Modification on Linear Range and Detection Limit

Electrode modification usually uses three main ways or methods, i.e.: (i) Mixing ink with a modifying agent; (ii) Electrochemical coating of the electrode surface; (iii) Coating the electrode surface by dripping nanoparticle material. Method (i) is performed before electrode printing on the substrate, and it has critical parameters related to the formulation of mixtures between substances. To make the molecules bond perfectly, the temperature during the curing process needs to be considered. At the same time, methods (ii) and (iii) are carried out after the electrodes are ready or printed on the surface of the substrate.

Electrode modifications are generally done to improve the selectivity and sensitivity of the immunosensor. The addition of electrons occurs on the electrode surface, especially when the electrode modification process is running. During transduction, the detection limit results obtained can also be more optimal than before the working electrode was modified. Figure 2 represents some examples of techniques for electrode modification.

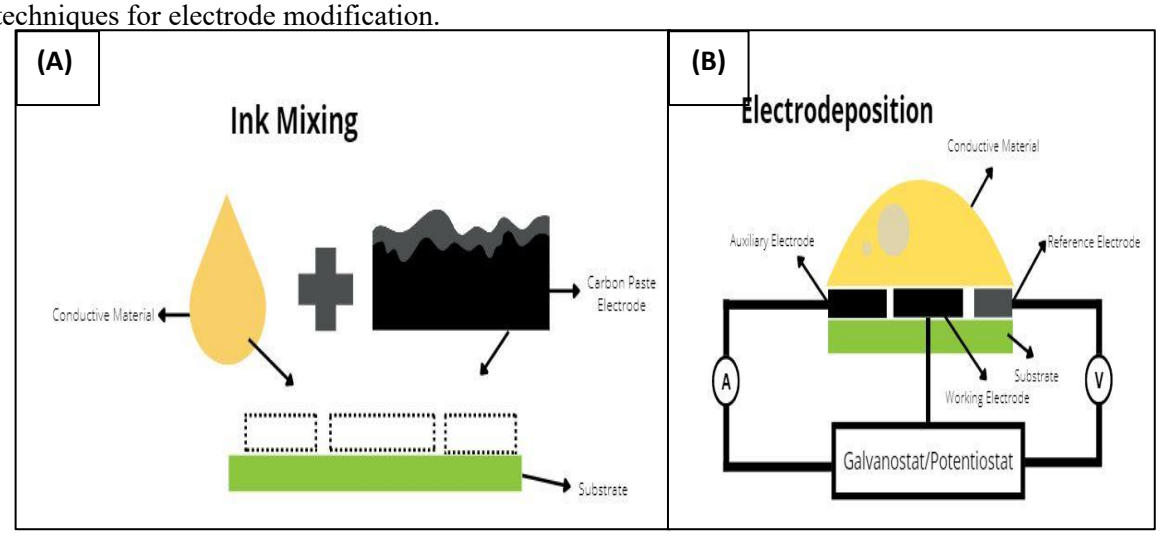

Table 2. Review of various immunosensor fabrications using electrode modification techniques.

Table 2. Electrode modification in the immunosensor fabrication process

NanoParticle	Modifikasi	Parameter	Analyte	Linear Range	LOD	Reference
MWCNT	Ink Mixing	40°C drying for 1 hour on paper	Aflatoxin B1 (AFB1)	1 ng/mL-30 ng/mL	0.62 ng/mL	(Migliorini et al., 2020)
Au	Drop Casting	20 µL; AuNP Graphene composite; room temperature	P53 Protein	20-1000 fg/mL	4 fg/mL	(Heidari et al., 2019)
Au	Drop Casting	10 µL; AuNRs-g-C_3N_4; DDried at room temperature	NS1	3-177 ng/mL	0.03 ng/mL	(Ojha et al., 2022)
Ag	Potentiostatic Electrodeposition	Chronoamperometry -1.2V for 120 seconds	Chlorite	3-100 µM	3 µM	(Bujes-Garrido et al., 2018)
Carbon	Potentiostatic Electrodeposition	Chronoamperometry - 0.2 V for 200 seconds	beta-amyloid (Aβ)	1 fg/mL-100 pg/mL	3.84 fg/mL	(P.-G. Wang et al., 2023)
Au	Galvanostatic Electrodeposition	*Chronopotentiommetry* -100µA, *for* 6000 seconds	Glukosa	0.01-5 mM	6 µM	(Núnez-Bajo et al., 2018)
Au	Galvanostatic Electrodeposition	*Chronopotentiommetry 30 nA, for* 50 seconds	Glukosa	0.5 µM-4 mM	0.07 µM	(Niu et al., 2013)
Carbon	Ink Mixing	0.4 graphite dan 0.1 cellulose acetate dissolved in acetone and cyclohexanone	CA15-3 antigen	0.5-200 U/mL	0.15 U/mL	(Hosseinzadeh et al., 2022)

There are three main components in the modification process with the ink mixing method such as (i) conductive particles, which are usually made of carbon material, (ii) binders so that particles can be transferred into the substrate, and (iii) modifying agents. In this case, NP metal. Some parameters that must be optimized in this method are the formulation of ink, Rheology when printing ink on SPE substrates, selection of substrate materials, and thermal curing for the drying process. Migliorini et al. (2020) conducted a study to detect Aflatoxin B1 (AFB1) from animal toxin samples. *Ink Mixing* modification is done by making conductive ink from the dissolution of MWCNT and Chitosan, which is then printed on paper and dried at 40 °C for 1 hour. The result is a linear range of 1 ng/mL to 30 ng/mL and a detection limit of 0.62 ng/mL

The drop casting method is the most common and easy method for electrode modification because only a few parameters must be optimized, including, for example, the volume of metal Nano Particles (NP) to be dripped or poured on the electrode or it can also be the concentration of metal NP in the solution. In this method, there are two ways to modify the electrode, namely modification by dripping directly with the metal NP substance on the electrode or by ex-situ fabrication where the composite made of NP is attached with carbonous nanomaterial first, then dripped or poured on the electrode. The direct dripping method is much easier and time-efficient in the process. Metal NP solutions that can be used include bismuth (Mayorga-Martinez et al., 2013), platinum (Popa et al., 2015), rhodium (Gatselou et al., 2015), gold (Jirasirichote et al., 2017), silver (Shamkhalichenar &; Choi, 2017), copper (Shabalina et al., 2017) and nickel (García & Escarpa et al., 2012).

When compared to ink mixing, the drop-casting method is easier and more affordable. But the thing to note in this method is that metal NP is only poured or dripped and dried on the working

electrode. Nanoparticles tend to aggregate when drying so that microparticulate will appear on the surface. Maintaining the temperature and pH of NP composites during storage is crucial to prevent aggregate formation during the electrode modification process (Jirasirichote et al., 2017). Overcoming the problem of the emergence of aggregates can be done using the second method, by synthesizing NP composites with carbonous nanomaterials.

This method is not only useful for the modification of working electrodes but will also increase the electroactive area based on the character of the carbonous nanomaterial. Materials that can be used for the synthesis method of metal NP and carbonous nanomaterials are Platinum (Chou et al., 2018), silver (Yao et al., 2016), nickel (Hjiri et al., 2015), and gold (Sadique et al., 2022). Research on modification with drop casting techniques was carried out by Heidari et al. (2019) by dripping 20 µL of gold volume on graphene composites. Immunosensors are used to detect P53 protein, with linear range results and detection limits of 20-1000 fg/mL and 4 fg/mL, respectively. Another research for electrode modification by drop casting technique was conducted by Ojha et al. (2022). Researchers dripped 10 µL of gold and left it at room temperature to detect dengue NS1. The results show a linear range of 3-177 ng/mL and a detection limit of 0.03 ng/mL The potentiostatic electrodeposition technique is performed by providing a fixed voltage value. When a specific voltage value is given, the NP metal composite coats the electrode surface. Its size and shape can be modulated as long as the electrode is submerged in the salt liquid. When the voltage becomes negative, there is an increase in the nucleation rate.

The smaller NP particles obtained will increase the electrode surface's electroactivity. Bujes-Garrido et al. (2018) applied this technique to detecting chlorite. The particle used for modification is silver which is depositioned by applying a voltage of -1.2 V for 120 seconds. The result is a linear range of 3-100 µM and a detection limit of 3 µM. Research on immunosensor fabrication with potentiostatic electrodeposition modification technique was also carried out by Wang et al. (2023) for detecting beta-amyloid (Aβ). In this research, carbon particles were deposited by applying a voltage of -0.2V for 200 seconds. The results show a linear range of 1 fg/mL-100 pg/mL and a detection limit of 3.84 fg/mL. The technique of electrical deposition can also be applied by providing a constant current or known as Galvanostatic Electrodeposition. Niu et al. (2013) detected analytes in the form of glucose with Ni particles given a constant current of 30 nA for 50 seconds. The result is a linear range of 0.5 µM-4 mM and a detection limit of 0.07 µM. Núnez-Bajo et al. (2018) did the same to detect glucose but by changing some parameters. In this study, researchers used Au particles as modifiers and a constant current of -100µA for 6,000 seconds to detect glucose. The result is a linear range and detection limits of 0.01-5 mM and 6 µM.

3.3. Fabrication Process based on Immunoassay configuration

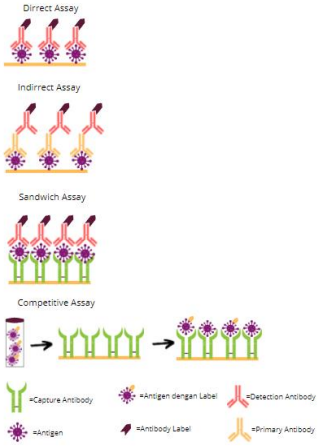

Figure 3. Immunoassay configuration

Immunoassay detection or testing is divided into several classifications, i.e., based on the label or without label and immune reactions. Labels are used as markers. If antibodies and antigens bind to labels, there is a significant increase in the number of electrons that has an effect when characterized. The current produced will be greater when compared to without using labels. The types of labels used include enzymes (Vargas et al., 2019), chromophores (Nakamura et al., 2019), fluorophores (Schreiber et al., 2021) or radioisotopes (Fukushima et al., 2021). Immunoassays are divided into four types based on the immune reaction configuration: direct, indirect, sandwich, and competitive. Figure 3 represents some techniques for configuring immunoassays.

Table 3. Immunoassay configuration in the immunosensor fabrication process

Electrode	Receptor	*Immunoassay*	Analyte	Linear Range	LOD	Reference
GCE/MWCNT	Fe^{3+}Hydrogel Alginate	Sandwich	Neuron specific Enolase	1pg/ml-100 ng/ml	0.447 pg/ml	(Yin & Ma, 2019)
GCE/Au@AgNP	Ab_1Solution	Sandwich	Carcinoembryonic antigen (CEA)	0.0001-100 ng/mL	0.005 pg/mL	(P. Chen et al., 2019)
SWCNT/SPCE	Larutan protein HIV 1 P24	Competitive	P24 antigens	10 pM-1 nM	2 pM	(Giannetto et al., 2017)
Surface Plasmon Resonance Chip	staphylococcal protein A	Direct	chlorpyrifos monoclonal antibody	0.25-50 ng/mL	0.056 ng/mL	(Q. Li et al., 2019)
Array carbon electrode	MERS-CoV antigen	Direct	MERS-CoV antibody	0.01-10,000 ng/mL	1 pg/mL	(Layqah & Eissa, 2019)
AuSPE	Anti-E.Coli	Sandwich	E Coli Bacteria	10^2-10^3 CFU/mL	30 CFU/mL	(Cimafonte et al., 2020)

Table 3 represents some of the results of research whose fabrication process contains the Immunoassay configuration process

Based on table 3, it is known that Yin & Ma (2019) conducted a study to detect Neuron Specific Enolase (NSE), which is a marker of patient brain injury due to hypoxia, ischemic, and trauma to the central nerve. A sandwich configuration was used in the study, where antibodies were bound to 〖Fe〗^(3+)Hydrogel Alginate by magnetic processing. Specific Enolase neurons are immobilized with antibodies bound to the substrate and then reattached with a secondary antibody (〖Ab〗_2) and labeled glucose oxide. The study results obtained a linear range and detection limits of 1 pg / mL – 100 ng / mL and 0.447 pg / mL. The Sandwich Immunoassay configuration is also used by Chen et al. (2019), where the primary antibody (〖Ab〗_1) is immobilized on the substrate. The CEA antigen is bound to the primary antibody, followed by the binding process of the secondary antibody (〖Ab〗_2), which has been bound by the label in the form of HRP on the substrate that has been mobilized with the primary antibody and CEA antigen. The results are 0.0001-100 ng/mL for the linear range and 0.005 pg/mL for the detection limit.

Fabrication of other immunosensors with the sandwich immunoassay technique was carried out by Cimafonte et al. (2020) to detect the target pathogen of E-Coli bacteria. The result is a linear range of 〖10〗^2 - 〖10〗^3 CFU/mL and a detection limit of 30 CFU/mL. The sandwich immunoassay technique is usually applied to antigens with more than two epitopes. In principle, the antigen of the sample is immobilized on the capture antibody that has been adsorbed on the WE. After that, the detection of the antibody that has been attached to the label is immobilized on the complex bond between the captured antibody-antigen.

Competitive immunoassay is a fairly complex technique. It is commonly used for small antigens with one epitope. Research on competitive immunoassay was conducted by Giannetto et al. (2017), where the binding process of capture antibodies (anti-p24) and p24 antigen in human blood samples were carried out by incubating with a temperature of 25°C for 1 hour outside the substrate. Then the solution containing antibodies and p24 antigens that have been bound is dripped on a substrate that has been mobilized with HIV protein solution 1 P24, only then incubated at room temperature for 1 hour along with an alkaline phosphate label that has been bound to secondary antibodies. The results show for a linear range of 10 µm-1 nM and a detection limit of 2 pM.

Direct immunoassay configuration can be done with or without labels. Suppose the test is carried out without using labels when the analyte in the form of antibodies is reacted with the adsorbed antigens on the surface of the Working Electrode (WE). In that case, it will form antigen-antibody complex bonds. When a complex bond is formed, there will be a change in the magnitude of the electric current signal when characterized. And without labels, the signal will be inversely proportional to the number of bound antigens. This means that the more the number of bound antigens, the smaller the current signal produced. Unlike direct immunoassay without labels, immunoassay testing carried out using labels requires an additional process in the form of attachment of substrates to antigen-antibody complex bonds after the process of forming antigen-antibody complex bonds. In this way, the magnitude of the signal produced is proportional to the number of antigens bonded. In general, the direct immunosensor process using this label takes a long time when the label attachment process with its analyte (Daniels & Pourmand, 2007).

Direct Immunoassay configuration research was conducted by Layqah & Eissa (2019) by immobilizing MERS and COV antigens, then samples with MERS or COV antibodies were dripped on electrodes and characterization by electrochemistry. The results obtained a linear range of 0.01-10,000 ng/mL and a 1 pg/mL detection limit. Then for, other direct immunoassay configurations were carried out by Q. Li et al. (2019) to detect targets in the form of chlorpyrifos monoclonal antibodies. Target concentrations are quantified based on optical shift or surface plasmon resonance due to weight changes in the substrate. The linear range obtained in the study was 0.25-50 ng / mL, and the detection limit was 0.056 ng/mL.

3.4. Analitnya Immobilization Techniques Based on the Type of Analyte

Immobilization is key in the Electrochemical Immunosensor (EI) fabrication process that uses molecular functional principles, where molecular conditions can be unstable, rare, and/or present at low concentrations. This paper will discuss five immobilization methods commonly used for the EI Sensor fabrication process, namely (i) Covalent Binding, (ii) Entrapment, (iii) Cross-Linking, (iv) Adsorption, and (v) Biological Binding. Immobilization can be done during the active surface formation process on the sensor or can also be done after the base layer is attached with a transducer. For example, the technique/method of immobilization, entrapment, and cross-linking is carried out by mixing active components with carriers and polymerizing agents by mixing directly during the fabrication process of transducers or electrodes to form an active surface on the sensor. As for the Covalent method, Adsorption and Biological Immobilization are carried out by binding active components such as enzymes, DNA / RNA, proteins, antibodies, and antigens on the surface of electrodes or transducers

that have been formed before. Each analyte and material on the electrode has its own characteristics, so it needs suitability for the immobilization process. Table 4 reviews immobilization techniques in the immunosensor fabrication process series.

Table 4. Immobilization techniques in immunosensor fabrication processes

Elektrode	Imobilisasi	Sample	Analytes	*Linear Range*	LOD	Reference
GCE	Adsorption	Food	Ochratoxin A	1-1,000 ng/mL	0.2 ng/mL	(X. Liu et al., 2013)
GCE	Adsorption	Food	Ochratoxin A	10^{-4}-20 ng/mL	0.01 ng/mL	(Viter et al., 2018)
GCE	Covalent	Serum	Anti-tTG IgA dan IgG	-	1.7 AU/mL (IgA) 2.7 AU/mL (IgG)	(Giannetto et al., 2014)
GCE	Covalent	Milk	Penicillin G	5.20-41.6 nmol/L	1.82 nmol/L	(Wu et al., 2013)
MWCNT	Entrapment	Protein Biomarker	Tumor Necrosis factor alpha	60-100 pg/ml	2.0 pg/ml	(Mazloum-Ardakani et al., 2015)

The adsorption technique is the easiest method to immobilize active molecules on the sensor surface. In general, adsorption is done by dripping or placing a molecular solution on the surface of the working electrode (WE), and the molecules will be adsorbed on the surface of WE at a certain time. Depending on the immobilization molecule, transducers may be hydrophilic or hydrophobic and may contain ionic groups. Some surface materials that can be adsorbed and often used are silica, cellulose acetate membranes, and polymers such as PVC polystyrene. This adsorption immobilization technique has several disadvantages, such as being prone to molecular leakage during the washing process and susceptible to changes in pH, temperature, and samples/media containing ionic. Liu et al. (2013) conducted a study to detect Ochratoxin A (OTA) from food samples. The analyte will be adsorbed directly on the electrode surface and bound to anti-OTA monoclonal antibodies after incubation treatment at 37°C for 30 minutes. The linear range and detection limit obtained is 1-1000 ng/mL and 0.2 ng/mL. Five years later, Viter et al. (2018) conducted research with the development of a photoluminescence immunosensor to detect OTA by immobilization of adsorption on GCE electrodes. The result is a better linear range of 〖10〗^(-4)-20 ng/mL and a detection limit of 0.01 ng/mL.

Covalent technique, where the bond between the electrode and the linker receptor is in the form of a chemical chain. Polymer detection or molecules in the Electrochemical Immunosensor (EI) can be covalently bonded directly to the transducer sensor or to the membrane or film on the transducer. Immobilization with this covalent bond method has several advantages, namely resistance to changes in PH, Ion strength, and temperature, and it is most stable for consumable use or repeated use (Smith et al., 2020). Giannetto et al. (2014) successfully detected Anti-tTG IgA and IgG in human blood serum

samples with a covalent technique with detection limits. The results obtained were 1.7 AU/mL and 2.7 AU/mL for IgA and IgG. While Wu et al. (2013) used a covalent immobilization technique to detect penicillin G in milk with a linear range result of 5.20-41.6 nmol/L, and the optimal detection limit that can be obtained is 1.82 nmol/L.

Entrapment techniques are often applied to immobilize biomolecules (glucose, urea, and mercury) in a series of biosensor fabrication processes. This technique is quite complicated to apply because it requires mixing receptors and analytes before printing on the substrate. Mazloum-Ardakani et al. (2015) successfully detected tumor necrosis factor-alpha by entrapment technique on MWCNT electrodes with 60-100 pg/mL results for the linear range and 2 pg/mL for the detection limit.

3.5. Techniques of Electrochemical Transduction in *Immunosensors*

In general bioelectrochemical reactions in Electrochemical Immunosensor (EI) can be known and measured based on current (amperometric) (Schachinger et al., 2021), voltage (potentiometric) (Ding &; Qin, 2020) and changes in conductivity values at electrodes or reagent medium (Conductometric) (J. Liang et al., 2018). In addition, other electrochemical detection techniques can also be applied by measuring impedance, both resistance and reactance (Impedimetric) (Hussein et al., 2021). Figure 4 is a schematic of electrochemical immunosensing.

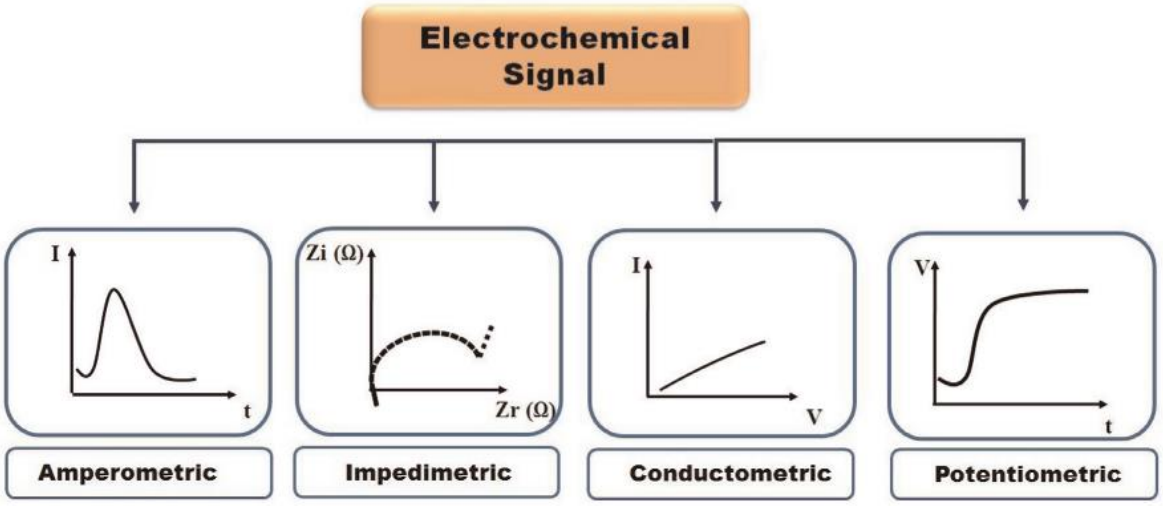

Figure 4. Electrochemical characterization (Burcu Bahadır &; Kemal Sezgintürk, 2015)

Research with various specific types of detection has been summarized in table 5 below.

Table 5. Types of detection on immunosensors

Electrode	Receptor	*Immunosensing*	Analyte	Linear Range	LOD	Reference
GCE/AuNP	Anti-CA125 monoclonal Ab	Amperometric	Cancer Antigen 125	0.0005-75 U/mL	6 µU/mL	(Samadi Pakchin et al., 2020)
GCE/AuNP	PSA Antibody	Amperometric	Prostate specific antigen (PSA)	100 fg/mL-50 ng/mL	0.03 pg/mL	(Dai et al., 2019)

Table 6. (Continued)

Electrode	Receptor	*Immunosensing*	Analyte	Linear Range	LOD	Reference
GCE/AuNP	anti-CEA antibodies	Amperometric	CEA Antigen	0.001–80 ng/ml	0.3 pg /mL	(W. Li et al., 2020)
Gold Disposable Electrode/CYS/ AU	Anti-NS1	Potentiometric	NS1	1100-10000 ng/ml	30 ng/mL	(Figueiredo et al., 2015)
Field Effect Transistor	Anti Influenza	Potentiometric	Avian influenza virus	$10^{0.5}$-$10^{8.5}$ TCID 50/mL	$10^{0.5}$ TCID 50/mL	(Hideshima et al., 2019)
CeFe-MOF	CA19-9 antibody	Impedimetric	CA 19-9	$0.0001 - 10\ U/mL$	0.00001 U/mL	(M. Wang et al., 2019)
Au/AuNP	Anti-CRH	Impedimetric	CRH peptide	$10 - 80\ \mu g/mL$	$2.7\ \mu g/mL$	(Duran et al., 2019)
PANI/ZnO nano composite film	E. coli serotype O157 mouse monoclonal antibody	Conductometric	Escherichia coli patogen	10-10000 CFU/mL	13.9 CFU/m L	(Mutlaq et al., 2021)

Amperometrics is a type of EI sensor that continuously measures current during oxidation and reduction processes in electroactive species in biochemical reactions (Hammond et al., 2016). The resulting current is proportional to the concentration of oxygen. The biochemical reaction process is carried out by measuring the reduction of oxygen in the Working Electrode (WE) against the reference electrode (RE), which is given a constant voltage (L. Liu et al., 2014). Samadi Pakchin et al. (2020) successfully fabricated immunosensors to detect CA 125 biomarkers in ovarian cancer. The type of detection used is amperometric, with GCE electrodes modified with nanoparticles in the form of gold and immobilized receptors in the form of anti-CA 125. The results obtained a linear range of 0.0005-75 U/mL and a 6 µU/mL detection limit. Previously Dai et al. (2019) also fabricated immunosensors with the same type of detection. Dai uses amperometric to detect prostate-specific antigen (PSA) present in human blood samples. The receptor used is the PSA antibody. The results show a linear range of 100 fg/mL-50 ng/mL and a detection limit of 0.03 pg/mL. W. Li et al. (2020) fabricated immunosensors with amperometric detection types to detect CEA antigens. The results were a linear range of 0.001-0 ng/ml and an optimal detection limit of 0.3 pg/mL.

In addition to amperometric detection types, potentiometric detection types are also commonly used. Potentiometric sensors are suitable for applications to measure low analyte concentrations at very small sample volumes. Research conducted by Figueiredo et al. (2015) used potentiometric immunosensors for dengue detection via NS1 antigen using biological bonding using IgY produced from egg yolk. The result is a linear range of 1100-10000 ng/ml and a 30 ng/mL detection limit. Then

the fabrication of immunosensors with the same type of detection was also carried out by Hideshima et al. (2019) to detect Avian influenza in FET applications. The linear range results are 〖10〗^0.5-〖10〗^8.5 TCID 50/mL, and the detection limit is 〖10〗^0.5 TCID 50/mL.

Another type of detection for immunosensor applications is impedimetric. The fundamental of the Impedimetric sensor is to provide a constant AC voltage. Then when there is a reaction between molecules at the electrode, there will be a change in frequency. Impedimetric immunosensors can be applied for the detection of target biomarkers and the formation of antibody-antigen bonds without the need for labels such as amperometric immunosensors. The response arising from changes in interfacial properties causes changes in resistance at the electrodes and in impedance due to immune recognition (Ho et al., 2009). M. Wang et al. (2019) used impedimetric immunosensors to detect tumor markers CA 19 – 9. The result is a linear range of 0.0001-10 U/mL and a detection limit of 0.00001 U/mL. Duran et al. (2019) also used impedimetric immunosensors to detect CRH peptide hormones. The result is a linear range and detection limits of 10-80 µg/mL and 2.7 µg/mL.

This type of conductometric detection describes the relationship between conductance and immune recognition. When the antibody that has been attached to the label in the form of an enzyme is conjugated with the antigen in the sample solution, there will be a change in the conductivity value of the electrolyte. The enzyme stops reacting due to the effects of the complex antibody-antigen bond that closes the electrode surface, so the concentration of ions in the electrolyte solution changes, and the current flow will also change (Z.-G. Chen, 2008). Conductometric immunosensors have the advantage that the voltage required is low and can be made with compact dimensions because, without a Reference, Electrode (RE) can still be used to detect targets in several applications (K.-Z. Liang et al., 2009). Research conducted by Mutlaq et al. (2021) uses conductometric immunosensors to detect E.Coli pathogens, where the receptor used is the E. coli serotype O157 mouse monoclonal antibody. The results obtained, the linear range is 10-10000 CFU/mL, and the detection limit is 13.9 CFU/mL.

4. Conclusion

Detection of analytes in the form of antigens, antibodies, biomarkers, and proteins with the principle of immunosensors is a very promising method because the concentration of analytes can be directly measured based on changes in voltage and current that occur during the process of reduction and electrochemical oxidation. This method is very effective compared to other methods, especially when compared to the ELISA method, which requires complex instruments because it must measure the absorbance of light, which must then be converted into electric current, then the simplification immunosensor can be developed as a Point of Care testing. However, each analyte has different characteristics, so a specific method is needed for labeling if the analyte cell character is not an electroactive cell. Then each analyte also requires a specific immobilization technique so that the analyte/target can bind properly to the electrode or receptor. The linear range and detection limit are also important parameters that can be developed so that very limited analyte concentrations in the sample can be detected. This paper does not describe the fabrication steps to achieve long stability. It is necessary to review the fabrication method to improve the stability of the immunosensor so that the ligands contained in the immunosensor electrode can last for a long time so that mass production can be carried out.

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