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Review article

Detecting epithelial sodium channel (ENAC) protein levels as a biomarker of hypertension

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ABSTRACT

Epithelial Sodium Channel (ENaC) is a protein that plays a role in maintaining sodium levels in the body. ENaC protein is abundant in the intestines, lungs, and kidneys. ENaC protein levels were measured from specific locations where the kidneys would be a good source of sodium excretion associated with hypertension. ENaC protein is one of the biomarkers hypertension based on lifestyle because it is related to sodium intakes in the body. ENaC protein is a biomarker of hypertension because it plays a role in sodium ion transport in cells which is related to blood pressure levels. Most hypertension or high blood pressures are not diagnosed on time because they are asymptomatic and existing protocols are less effective. The method to detect ENaC protein has been developed and can be a solution to this problem. This review describes various methods to detect ENaC protein as a biomarker of hypertension.

Keywords: ENaC protein, Sodium, Biomarker, Hypertension.

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INTRODUCTION

Epithelial Sodium Channel (ENaC) is a protein that functions as an ion channel bound to a permeable membrane that is selective for Na^+ ions consisting of four subunits namely α, β, γ, and σ [1]. ENaC protein is abundant in the lungs, intestines, and kidneys. In the kidney, ENaC protein is related to sodium intake in the body and is associated with hypertension and is responsible for regulating sodium balance, blood volume, and blood pressure [2].

ENaC protein is one of the biomarkers of many biomarkers of hypertension based on lifestyle because it is related to sodium intake in the body. Sodium intake from salt is one of the risk factors for hypertension that can be controlled. WHO states that reducing salt intake to less than 2 g / day will reduce blood pressure, the risk of cardiovascular disease, stroke, and coronary heart disease [3]. However, the Indonesian Ministry of Health concluded that the average salt consumption in Indonesia is 3.6 g/day. Thus, the high consumption of salt may not be realized by the Indonesian people, so that it can be one of the most significant contributors to hypertension in Indonesia [4].

Based on data from the World Health Organization (WHO), nearly 1 billion people worldwide have high blood pressure. Hypertension is one of the leading causes of premature death worldwide. Approximately 40% of adults suffer from hypertension which is not diagnosed in a timely manner because it is asymptomatic. Therefore, hypertension is often referred to as the silent killer. Based on these data, it is shown that the established protocol for diagnosing hypertension is not effective, but timely diagnosis is more difficult [5].

Detection of hypertension associated with high sodium levels can be done by determining the amount of ENaC protein in the patient's body. The higher sodium concentration, the higher activity of the ENaC protein in absorbing sodium. It has been shown that ENaC expression promotes Na^+ retention, leading to an increase in blood pressure [1]. This review describes the various methods that have been developed to detect ENaC protein as a biomarker of hypertension, namely the Enzyme-Linked Immunosorbent Assay (ELISA) [6], electrochemical immuno sensor [7], electrochemical APTA sensor [8], electrophoresis [9], and western blot [10].

HYPERTENSION

Hypertension is one of the most dangerous diseases in the world and a major risk factor for heart attacks [11], strokes [12], and kidney failure [13]. Most cases of hypertension have no known cause, but conditions associated with an increased likelihood of a person suffering from hypertension include heredity, increasing age, unhealthy lifestyles such as alcoholic beverages, consuming foods high in salt, consuming caffeine, obesity, stress and smoking [4,5].

Hypertension is characterized by an abnormal increase in blood pressure, both systolic blood pressure and diastolic blood pressure. Hypertension or called high blood pressure is a condition where the

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systolic blood pressure is ≥140 mmHg and diastolic ≥90 mmHg. Meanwhile, at normal blood pressure, systolic blood pressure <120 mmHg and diastolic <80 mmHg. Hypertension occurs due to increased fluid in the circulation caused by abnormalities of kidney function so that it is not able to get rid of a certain amount of salt and water in the body, so when the volume of blood in the body increases, blood pressure also increases so that a person gets hypertension more easily [14,15].

To determine the patient's blood pressure, an examination is usually carried out using a sphygmomanometer and supporting examinations, such as urine tests, blood tests, cholesterol tests and heart record tests. Efforts to prevent and control hypertension can be done by improving a healthy lifestyle and increasing public awareness [16].

Examination of biomarkers that specifically indicate the pathological mechanism of disease severity and response to therapy can be an ideal substance in the management of hypertension. Therefore, early detection of hypertension is carried out by observing specific biomarkers in body fluids so as to minimize the risk of organ damage [17]. Hypertension can be detected through biomarkers in the patient's body, one of the hypertension biomarkers is protein Epithelial Sodium Channel (ENaC) [18].

Hypertension can be classified into two types, as follows [19]:

Based on the cause, divided into two

a. **Primary hypertension / essential hypertension:** hypertension whose cause is unknown (idiopathic), although it is associated with a combination of lifestyle factors such as lack of movement (inactivity) and diet. Occurs in about 90% of patients with hypertension [20].

b. **Secondary hypertension / non - essential hypertension:** hypertension with known cause. In about 5-10% of people with hypertension, the cause is kidney disease. In about 1-2%, the cause is hormonal disorders or the use of certain drugs (eg birth control pills) [20].

Based on the form of hypertension

Diastolic hypertension (diastolic hypertension), mixed hypertension (elevated systolic and diastolic hypertension), systolic hypertension (isolated systolic hypertension).

EPITHELIAL SODIUM CHANNEL PROTEIN (ENaC)

ENaC protein is an important substance in maintaining sodium levels. ENaC protein levels were measured from certain locations where the kidneys would be a good source for sodium excretion associated with hypertension [6]. The total amount of sodium that can be absorbed determines the extracellular fluid volume and blood pressure. Thus, although many systems can affect blood pressure in the short term, long term regulation of blood pressure ultimately depends on the handling of sodium in the kidneys. The sodium transporters in the kidneys are responsible for sodium reabsorption and fluid balance. Therefore, ENaC is an important candidate for its involvement in the development of hypertension [21]. ENaC dysfunction is directly associated with several disease states including Liddle syndrome, pseudo hypoaldosteronism, cystic fibrosis, and hypertension [22]. ENaC protein is used as one of the biomarkers of hypertension because

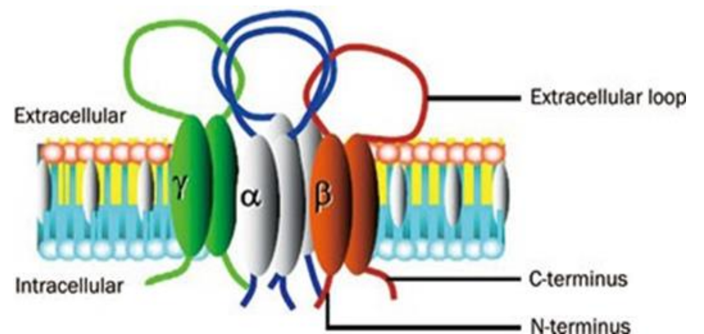
a. ENaC functions as a major channel in controlling the rate of renal sodium reabsorption where the control of Na^+ movement in epithelial cells is very important for regulation or homeostasis of extracellular fluid volume, electrolyte balance, and long-term blood pressure [23].

b. A genetic defect that causes hyperactive ENaC to form a monogenic hereditary form of hypertension in humans (Liddle's syndrome). This suggests that sensitivity to salt may arise from an imperceptible impairment of ENaC function and/or regulation.

The ENaC protein has 4 genes, namely SCNN1A, SCNN1B, SCNN1G, and SCNN1D in the human genome, each of which is responsible for encoding 4 subunits, namely α, β, γ, and δ. The ENaC α, β, and γ subunits share approximately 30% homology at the amino acid level, and each subunit has a molecular mass of 70–85 kDa. The -α subunit is essential for the formation of pores in absorbing ions, while the β and γ subunits are required to maximize channel activity and can play a regulatory role [24]. The δ subunit of ENaC will combine with the and subunits to form a functional channel [22].

Expression of the α, β, and γ subunits was found in the kidney, lung, and large intestine. However, the expression of δ subunits in the brain, pancreas, testes, and ovaries is known to be higher than in the kidneys and lungs. Expression of the δ subunit has also been detected in human nasal and ocular epithelium. Overall, the distribution of δ subunits in tissues is clearly different from that of α, β, and γ subunits [1].The three subunits work together to form a pore channel (Figure 1) [24].

Figure 1: Structure of the protein ENaC in the kidney



ENaC is located mostly in the epithelium as an active channel, ENaC allows the flow of Na^+ ions from the lumen into the cell epithelium across the apical cell membrane. The Na^+ ions

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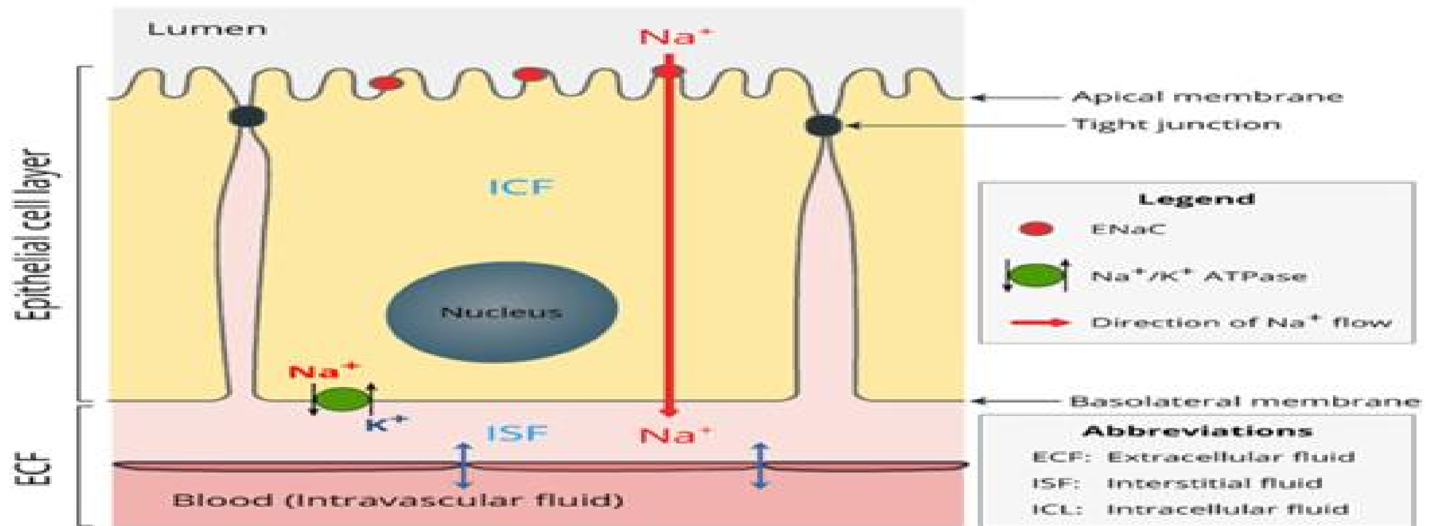
absorbed are then pumped out of the cells into the interstitial fluid by the activity of Na^+/K^+ ATPase located on the basolateral membrane. Since ENaC modulates the amount of Na^+ in the extracellular fluid, it has an important role in the regulation of extracellular fluid volume and blood pressure as observed in Figure 2 [1].

Figure 2: Schematic illustration of the location and function of ENaC in epithelial cells



METHOD OF DETECTING ENAC PROTEIN LEVELS AS BIOMARKER OF HYPERTENSION

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assay (ELISA) is a technique based assay plate / plate designed for the detection and quantification of peptides, proteins, antibodies, and hormones. In ELISA, the antigen must be immobilized onto a solid surface and then an antibody that binds to the enzyme is added. Detection is carried out by assessing the activity of the enzyme conjugate through incubation with the substrate to produce a measurable product. An important element in the detection strategy of ELISA is the antigen-antibody specific interaction. The reactants from the ELISA assay were immobilized into the microplate surface making separation of the unbound material easier. The ability to wash nonspecific non-bonded materials makes the ELISA assay an accurate tool for measuring specific analytes [25].

ELISA is one of the methods in the laboratory field, especially immunology to determine protein expression, immune reactions, and immune responses. In the field of immunology this technique is used to determine the presence of antigens or antibodies in samples/serum. The basic principle of the ELISA reaction is to react antigen with an antibody labeled with an enzyme which is then added with a substrate so that it will be hydrolyzed into a color precipitate that can be detected using an Elisa reader. At the final stage of the Elisa Technique, a stop solution is always added to stop the reaction. Strong acids are usually used as a stop solution [26].

Broadly speaking, there are two types of ELISA techniques, namely competitive and non-competitive. Competitive techniques use antibody-enzyme or antigen-enzyme conjugates. The non-competitive technique uses primary and secondary antibodies. Secondary antibodies in non-competitive techniques are conjugated with enzymes that function as signals. ELISA technique innovation has developed rapidly, the goal is to get optimal results. The following is an example of an ELISA technique innovation [25,26].

a. Direct ELISA

ELISA The direct ELISA technique is the simplest. In this technique it is usually to measure the antigen in the sample using a specific monoclonal antibody. An enzyme-labeled antibody is used, resulting in binding to an enzyme-labeled antibody-antigen complex. The advantage of this technique is that it only uses one antibody so it is fast and avoids cross reactions. Meanwhile, the disadvantages of this technique are that it is less sensitive and does not have the flexibility of enzymes [27].

b. Indirect ELISA

In the indirect ELISA technique, antibodies are sought so that specific antigens are needed. In this indirect ELISA technique, a specific antigen (monoclonal) is required, the antibody to be searched for in the sample, a secondary antibody labeled with an enzyme, a substrate, and a stop solution. The advantages of this technique are that it is more sensitive and has many secondary antibody variations. Meanwhile, the drawbacks of this technique are the non-specific antigen immobilization method and the long testing time [27].

c. Sandwich ELISA

The Sandwich Elisa technique is to look for the desired antigen and in the Sandwich Elisa, the sought antigen does not need to be purified. The Elisa Sandwich technique uses a primary antibody to react with the desired antigen in the sample and reacts with an enzyme-labeled secondary antibody. The antigens are multivalent such as polysaccharides or proteins that have at least 2 antigenic sites in order to interact with specific primary antibodies and specific

secondary antibodies labeled with enzymes [28].

d. ELISA Biotin Streptavidin

The level of sensitivity of this type of Elisa is relatively high. The working principle of this technique is the same as that of Elisa Sandwich, but uses optional primary and secondary antigens if the antibody sought in the sample does not react with the enzyme label.

e. Multiplex immunoassay

Multiplex immunoassay is the development of the principle of the Elisa Technique which aims to simultaneously test various analytes at the same time in one sample. The advantage of this technique is that it can measure the desired marker at the same time. The drawback is that it requires more sophisticated tools at a higher cost when compared to conventional Elisa.

f. Competitive ELISA

The Competitive ELISA technique is the development of the Elisa Technique which is useful for testing the presence of antigens or antibodies. The working principle of this technique is to add competitors to react with antigens or antibodies that occur in the microtiter. Elisa Competitive technique has a high level of sensitivity due to the specificity of antibodies and antigens. Therefore, the advantage of this technique is that the sample solution containing antibodies or antigens does not need to be purified [29].

The measurement of ENaC levels in various urine samples from three groups of patients: non-hypertensive, hypertensive with and without history, using the ELISA method. ENaC protein was detected in the supernatant and urine pellet after centrifugation. The results showed ENaC protein levels in non-hypertensive patients were 1.12 ng/mL, hypertensive patients with a history of 2.7 ng/mL, and hypertensive patients without a history of 4.0 ng/mL. These results suggest that the concentration of ENaC in urine samples may be associated with hypertension [6].

Electrochemical Immunosensor

Generally, ENaC protein can be detected in urine samples using the ELISA method. However, the ELISA method is time consuming and relatively complicated. Detection of ENaC using optical immunosensor in platelet samples was reported recently. The sensor was developed using an indirect immunofluorescence detection assay of GNPs-anti-ENaC bound to a fluorophore-labeled secondary antibody. This method can be used to differentiate between normotensive and hypertensive samples. Electrochemical biosensors are an ideal alternative method that provides a fast and easy procedure with a high degree of selectivity and sensitivity [18].

Biosensors can be classified according to biological recognition elements, one of which is immunosensor. Immunosensor is a biosensor based on the interaction between antibody and antigen on the surface of the transducer. Antibodies can be species that are immobilized on the transducer to detect the antigen. Due to the strong binding forces between these biomolecules, immunosensors present high selectivity and very high sensitivity, making them very attractive for many applications in different fields of science [30].

Based on the signal transduction mechanism, immunosensors can be classified into optical immunosensors, piezoelectric immunosensors and electrochemical immunosensors [31].

a. Optical Immunosensors

Optical sensors use light as a stimulus and are capable of detecting changes in light intensity as it passes through or refracts a sampling system that binds to antibodies or antigens. One example of an optical immunosensor is Surface Plasmon Resonance (SPR), a sensor-based.

b. Piezoelectric Immunosensors

Piezoelectric devices convert physical or mechanical changes into electrical energy and vice versa. One of the piezoelectric immunosensors is Quartz Crystal Microbalance (QCM).

c. Electrochemical Immunosensor

Electrochemical immunosensor is an analytical device that combines the interaction of specific antibodies or antibody fragments as identification molecules against a specific analyte (antigen) with high sensitivity from electrochemical transduction. Electrochemical immunosensors have become an interesting subject for clinical diagnosis, environmental monitoring and food analysis [31].

Electrochemical immuno sensors explore the measurement of electrical signals produced in electrochemical transducers. This signal can be voltametric, potentiometric, conductometric or impedimetric. Immune Reactions in electrochemical transducers can cause changes in potential, current, ion concentration, conductance, capacitance, or impedance [30].

On the research of detection ENaC protein was carried out using the electrochemical immunosensor method. This study used an electrochemical immunosensor based on gold nanoparticles-bioconjugate to detect ENaC and applied it to detect ENaC in urine samples. The bioconjugate (AuNP/HS-PEG-COOH/Anti-ENaC) was immobilized on the surface of the electrode Screen Printed Carbon Electrode gold which was treated with cysteamine (SPCE/ Au-cys), the -NHgroup$_2$ of cysteamine would interact with the terminal -COOH group of the bioconjugate via covalent bond. Bovine Serum Albumin (BSA) was added to the SPCE-bioconjugate surface to block and avoid nonspecific binding to the SPCE surface. Anti-ENaC in the bioconjugate will bind to the ENaC protein through antigen-antibody interactions. Schematic of this immunosensor is shown in Figure 3 [18].

The interaction of ENaC with anti-ENaC in the bioconjugate was detected by DPV using a ferricyanide redox system. Tests were optimized using the Box-Behnken experimental design. The results showed that SPCE/Au increased the ferricyanide

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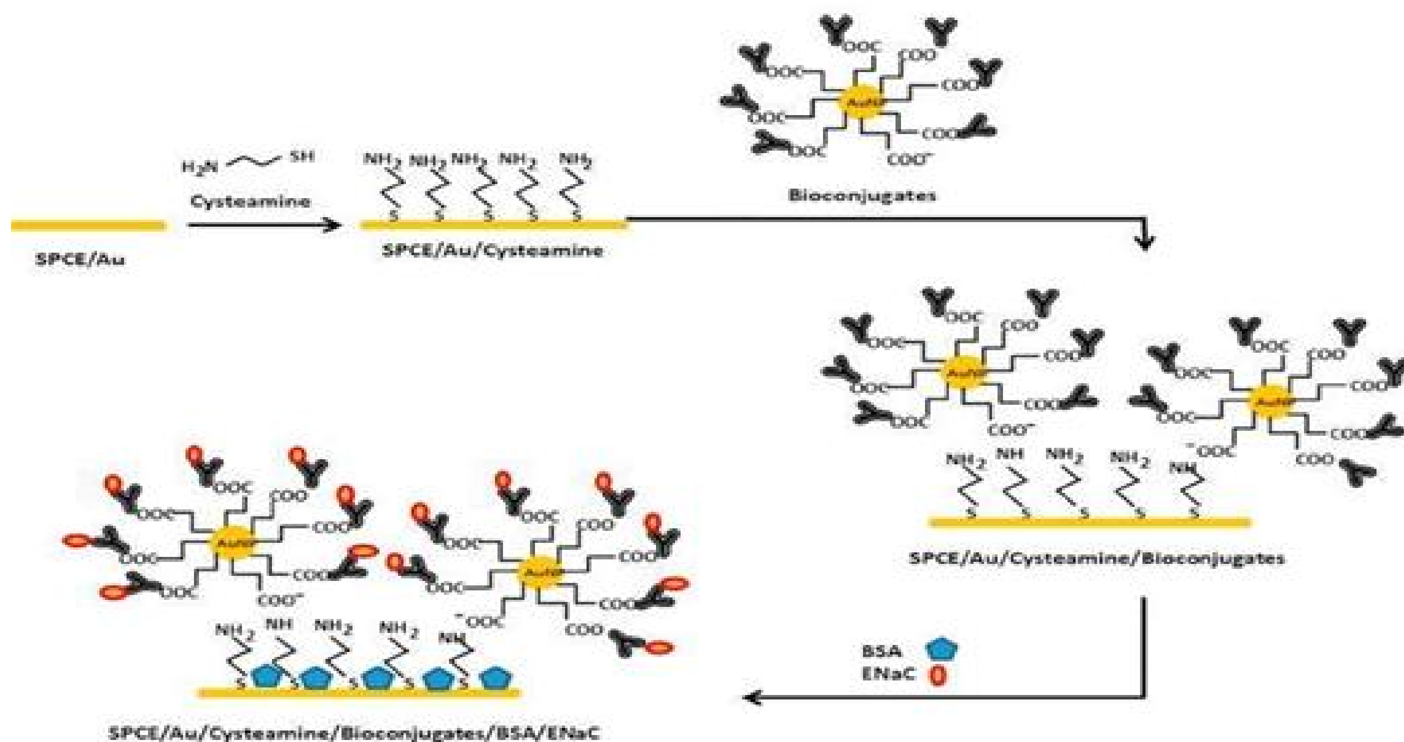
peak current signal 10 times compared to bare SPCE. The detection limit obtained was 0.065 ng/mL and the quantification limit value was 0.197 ng/mL for the range of 0.09375 to 1.0 ng/mL. So that the immunosensor method can be used to determine the concentration of ENaC protein in urine samples of hypertensive or non-hypertensive patients [18].

Figure 3: Schematic of the ENaC protein immunosensor



Electrochemical APTA sensors

Determination of ENaC protein levels in urine samples based on antibody methods that have been reported include the Enzyme-Linked Immunosorbent Assay method, which shows that ENaC protein can be measured in supernatant and centrifuged urine sediments, and electrochemical immunosensors from urine samples can measure ENaC without centrifugation and special treatment. However, antibody-based methods have several disadvantages, such as being relatively expensive, and can only bind to large molecules. Therefore, other alternative methods were developed, such as the determination of the protein content of ENaC through its interaction with the aptamer [8].

Biosensor with an aptamer identifier element is referred to as an APTA sensor. Aptamers are single-stranded RNA or DNA synthetic oligonucleotides selected through a method in vitro known as Systematic Evolution of Ligands by Exponential enrichment (SELEX) which can bind to its target with high affinity and specificity due to its 3-dimensional structure. The advantages of aptamers over antibodies are high stability, easy synthesis and modification, low immunogenicity and the ability to recognize a wide variety of molecules. APTA sensors are generally divided into three, namely electrochemical, optical, and mass sensitive. Aptamer has been used as an assay biosensor in various targets [32].

ENaC protein detection research using the APTA sensor method has been carried out. An electrochemical sensor for detecting ENaC was developed using SPCE electrodes modified with cerium oxide electrodeposition. The aptamer immobilization technique was performed using the streptavidin-biotin system. Streptavidin was immobilized on the SPCE/CeO_2 surface by passive adsorption; It is expected that the active group on streptavidin will interact with CeO_2 on the SPCE surface. BSA was added to the SPCE/CeO_2/streptavidin-biotin aptamer surface to block and avoid nonspecific binding to the SPCE surface. The ENaC protein will bind specifically to the ENaC aptamer that has been immobilized on the SPCE surface.

The schematic of this APTA sensor is shown in Figure 4 [8]. The interaction of ENaC with the ENaC aptamer was detected by DPV using a ferricyanide redox system. Tests were optimized using the Box-Behnken experimental design. The results showed that the Box-Behnken experimental optimization design for streptavidin incubation time, aptamer incubation time, and streptavidin concentration were 30 minutes, 30 minutes, and 10.8 g/mL, respectively. Various concentrations of ENaC were used to obtain a linearity range of 0.05-3.0 ng/mL, and the detection and quantification limits were 0.012 ng/mL and 0.038 ng/mL, respectively. This APTA sensor method has the potential to measure ENaC protein levels in urine samples as well as point-of-care devices [8].

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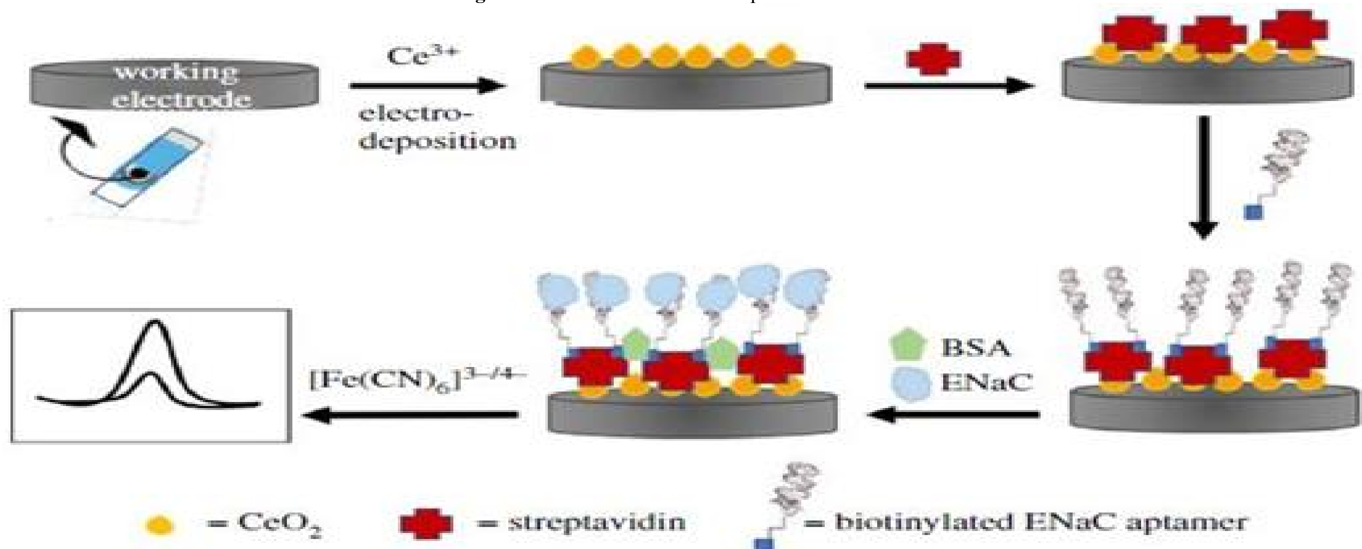
Figure 4: Schematic of the ENaC protein APTA sensor



Western Blot

Western Blot (WB) is a molecular biology technique for semi-quantitative analysis of protein expression of various test materials from both cells and tissues with its working principle based on antigen-antibody binding [33]. The stages of the WB technique are sample preparation (extraction and purification of protein, measurement of concentration, and the amount of protein to be used), separation of proteins using polyacrylamide gel electrophoresis, transfer of proteins from gel to nitrocellulose membrane or PVDF (Polyvinylidene Difluoride), membrane blocking, incubation primary and secondary antibodies, detection of target proteins, calculation of protein band intensity using digital densitometry software, and normalization using internal protein controls [34].

Western blot is widely used to identify and quantify specific proteins in complex mixtures. This technique allows indirect detection of protein samples immobilized on nitrocellulose membranes. Protein samples were first run with SDS – PAGE and electrophoretic transferred to the membrane. After the blocking step, the membrane was probed with primary antibodies, both monoclonal and polyclonal, which increased in number compared to the antigen. After sequential washing, the membranes were then incubated with secondary antibodies conjugated with antibody-reactive enzymes. Finally, the membrane is re-washed with the substrate of the appropriate enzyme, which then produces a recordable signal [35].

Studies on the increased expression of ENaC protein in neutrophils from hypertension patients have been carried out. The WB assay was used to demonstrate the presence of a phosphorylated form of -ENaC compared to neutrophils from healthy individuals. Although neutrophils from circulating hypertensive subjects in an activated state exhibit oxidative stress and modifications noted by confocal, atomic force, and scanning electron microscopy. Neutrophil activation involves phosphorylation of several proteins, which in turn regulate chemo taxis, F-actin assembly, and signaling [10].

Electrophoresis

Electrophoresis is a chemical analysis method based on the movement of charged protein molecules in an electric field (isoelectric point). The movement of molecules in an electric field is influenced by the shape, size, and magnitude of the charge and chemical properties of the molecule. Separation is carried out based on differences in the size of the molecular weight and the electric charge contained by the macromolecules. When an electric current is applied to a buffer medium that already contains plasma proteins, the protein components will begin to migrate [36].

Electrophoresis is a method for separating large molecules (such as proteins, DNA fragments, RNA) by using an electric field that is applied to a medium containing the sample to be separated. This separation of charged molecules is based on the difference in their rate of migration in an electric field. This technique can be used by exploiting the electric charge present in macromolecules. If a negatively charged molecule is passed through a medium, then the molecule will move from a negative charge to a positive charge. The speed of motion of the molecule depends on the ratio of charge to mass and the shape of the molecule [37].

Electrophoresis is divided into two types, namely paper electrophoresis and gel electrophoresis [36].

a. Paper Electrophoresis

Paper electrophoresis is a type of electrophoresis consisting of paper as the stationary phase and dissolved charged particles (mainly complex ions) as the mobile phase. The movement of particles in paper depends on the charge or valence of the solute, cross-sectional area, applied voltage, electrolyte concentration, ionic strength, pH, viscosity, and solute absorption [38].

b. Gel electrophoresis

Gel Electrophoresis is a technique for separating a macromolecule by applying a force to the macromolecule to pass

through a medium containing a gel which is assisted by electric power. This electrophoresis uses a gel as the stationary phase to separate molecules such as DNA and proteins into bands each of which are molecules of the same length [39].

Protein electrophoresis is basically carried out on the same principle as that used in DNA electrophoresis, but the gel used is a polyacrylamide gel. The most common protein separation method is by electrophoresis using discontinuous polyacrylamide gel as a buffer and sodium dodecyl sulphate (SDS) to denature protein. Such electrophoresis method is called SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis). SDS is an anionic detergent which when dissolved its molecules have a negative charge in a wide pH range. The main function of SDS in methods other than to denature proteins is to give a negative charge to the protein to be analyzed. Meanwhile, acrylamide in the SDS-PAGE method serves to prevent diffusion due to heat generation in electric currents. In addition, acrylamide gel is also used to separate small protein molecules.

Based on research that has been carried out on the use of constant denaturing capillary electrophoresis to identify single nucleotide polymorphisms in the genes (Scnn1a and Scnn1b) that encode and subunit ENaC protein. Screening of COOH mutations in the and subunits of the ENaC protein was performed. Blood samples from 184 individuals from 31 families who participated in the study on the genetics of hypertension were analyzed. Exon 13 of Scnn1a and Scnn1b, which encode the COOH second and termini transmembrane segments of an ENaC, respectively, were amplified from DNA samples collected from members of each family by PCR. Constant denaturant capillary electrophoresis (CDCE) was used to detect mutations in the PCR products of the collected DNA samples [9].

CONCLUSIONS

Hypertension can be detected through biomarkers in the patient's body, one of the hypertension biomarkers is protein Epithelial Sodium Channel (ENaC). Early detection of hypertension is done by observing specific biomarkers in body fluids, so as to minimize the risk of organ damage. ENaC protein detection methods in recent years have been widely developed. Several ENaC protein detection methods have been developed, namely Enzyme-Linked Immunosorbent Assay (ELISA), electrochemical immuno sensor, electrochemical APTA sensor, western blot, and electrophoresis. The five methods can detect the ENaC protein with its respective advantages and disadvantages that have the potential to be further developed in the future.

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