PHOTOELECTROCHEMICAL DETECTION OF DENGUE-RELATED OLIGONUCLEOTIDE SEQUENCE USING ANTHOCYANIN AS A INTERCALATING AGENT AND ELECTROCHROMIC MATERIAL

Aryo Tedjo[1*)], Anwar S. Ibrahim[2], Kusmardi[3]

1. Department of Medical Chemistry, Faculty of Medical Science, Universitas Indonesia, Jakarta 10430, Indonesia
2. Department of Medical Physics, Faculty of Medical Science, Universitas Indonesia, Jakarta 10430, Indonesia
3. Departement of Anatomical Pathology, Faculty of Medical Science, Universitas Indonesia, Jakarta 10430, Indonesia

*)*E-mail: aryo.tedjo@ui.ac.id*

Abstract

World Health Organization (WHO) presupposes a confirmation of dengue virus infection diagnosis with two criteria, i.e. clinical and laboratory criteria. One of the basic methods used by most laboratories to diagnose dengue virus is to detect oligonucleotide sequence using a DNA amplification technique. In this research, the measurement of dengue-related oligonucleotide was conducted by photoelectrochemical method. The presence of oligonucleotide sequence in target DNA can be detected by DNA probe that is immobilized on TiO_2 electrode. The DNA hybrid is then bound to electrochromic substance like anthocyanin that generates current when it is subjected to light. The photocurrent is directly proportional to the number of target DNA. The aim of this research is to obtain photoelectrochemical system that has sensitivity and high responsiveness toward the change in oligonucleotide concentration, especially the applicability of anthocyanin as a electrochromic substance and intercalating agent. Linearity (R2) generated from the change of current in response to concentration changes of target DNA (in the concentration range of 0.75–3.00 nM) is 0.9611. Thus, this method has the potential to be developed to detect the presence of dengue virus in biological sample.

Keywords: anthocyanin extract, dengue-related oligonucleotide, photoelectrochemical

Introduction

In Indonesia, contagions that become the priority for prevention and eradication in the Millenium Development Goals (MDGs) are HIV/AIDS, malaria, and tuberculosis.[1] However, Dengue Hemorrhagic Fever (DHF) is also crucial to be eradicated because it has become an annual epidemic with hundreds of people dying every year.[2,3] The effect of DHF can also be more devastating than AIDS cases because apart from directly killing people, the symptoms are difficult to recognize and patients become too late to cure.[4]

The DHF's morbidity and mortality rate in Indonesia is quite high (number two after Thailand during 1985-2004).[3] This condition requires maximum handling including early detection. The diagnosis is not sufficient based on clinical syndrome; thus, it must also be supported by laboratory diagnosis. It is considered important because more than half of the infected show that asymptomatic condition or the fever is not differentiated.[5,6] Consequently, developing a method of dengue virus detection at an acute phase is needed to obtain a good treatment, etiology analysis, and correct and quick disease control.

According to WHO, to conduct a diagnosis of dengue virus infection needs clinical and laboratory criteria.[7] There are three basic methods that are used by most laboratories to diagnose dengue virus. These methods are the isolation and characterization of viruses, the detection of dengue-virus-specific antibodies, and the detection of oligonucleotide sequences using DNA amplification techniques with Polymerase Chain Reaction (PCR). Currently, molecular diagnosis of oligonucleotide sequences is conducted by reverse transcription PCR (RT-PCR), nucleic-acid-sequence-based amplification (NASBA), or real-time PCR (RT-PCR). Those methods slowly begin to replace virus isolation method as a new standard in the detection of dengue virus at the acute phase serum.[8]

One method of oligonucleotide sequence detection that has developed recently is the photoelectrochemical method. The photoelectrochemical method is reported to be applicable to determine nanoMolar DNA

concentration so that it is a very sensitive method for quantitative DNA analyses.[9-11] The advantage of using this method is comparable to that of using a quantitative analysis of DNA with RT-PCR, microarray, or FISH.

Other than being sensitive, this method is also simple because it does not need complicated data interpretation. Also, it is relatively inexpensive in the use of materials because it only requires a few chemicals, and it is easy to operate because it does not need special skills such as PCR or electrophoresis. Making a quantitative DNA analysis with this technique is a challenge that continues to develop, especially on things that are related to the use of surface electrode, electrochromic materials election, labeling technique, and especially its development prospect for biochip application.

Photoelectrochemical system is composed of two electrodes: electrochromic materials and electrolyte. One electrode is coated with a DNA probe that has been labeled with electrochromic material (dye). Electrochromic material is one that changes its color when given a variety of electrical fields or electric currents. This process is reversible and can be oxidized or reduced by electrochromic material and electron flow which occurs when it absorbs light. Some materials have the electrochromic property such as elements of the transition metals (tungsten oxide-WO_3, iridium oxide-IrO_2), organic materials (viologen, anthocyanin, anthraquinone, and pirazolin,), complex inorganic compounds (Prussian Blue - $Fe_3Fe(CN)_6$, methylene blue), and conductive polymer materials such as polypirrol, polyaniline, and polythiophene.[12]

The principle of this method can be described as follows: when the target DNA is inserted in the system, it is hybridized with immobilized oligonucleotide capture probe on TiO_2 electrode. Then if the system is given light through transparent electrode surface, there will be an oxidation-reduction reaction (redox) in electrochromic materials. Because electrochromic materials are bound to the DNA probe, the current will be also proportional to the concentration of target DNA.

One technique in photoelectrochemical methods that can be used to detect the presence of oligonucleotide sequences is the DNA intercalation technique. The intercalation of DNA occurs by stacking base pairs in the double stranded DNA molecule and polycyclic aromatic compounds. Some polycyclic aromatic compounds known as DNA intercalating agents are naphthalene, 3-methylcholanthrene, 7,12-dimethylbenz(a)anthracene, 1,2-benzanthracene, quinolizinium derivatives, resveratrol, genistein, and daunomycin.[13-16] In the DNA Intercalation technique, the target DNA hybridized with DNA probe is bound by intercalating agents which are also electrochromic materials. The advantage of using this technique is that there is no labeling of DNA probe with electrochromic materials because the DNA double helix (after hybridization between probe and target DNA) will bind directly with the intercalating agents.

Anthocyanins have very similar structures to potent intercalating agents such as actinomycin D and mitomycin C, which are used clinically. From previous studies, we had known that anthocyanins have the ability as DNA intercalating agents. On the other hand, we had also known that this material can improve the sensitivity of TiO_2 electrode.[17,18]

The aim of this research is to obtain a photoelectrochemical system that has sensitivity and high responsiveness toward the change of dengue-related oligonucleotide concentration, especially the applicability of anthocyanin as an electrochromic substance and intercalating agent.

Methods

This research is divided into three phases. The first phase is testing some electrochromic organic materials as intercalating agents. The organic materials are extracted with ethanol from several fruits to obtain colouring pigment such as anthocyanin. The second phase is testing the photolectrochemical system that uses DNA double helix, electrode, and electrochromic organic materials that have been determined at the first phase. The third phase is the use of photolectrochemical to measure dengue-related oligonucleotide concentration.

Preparation of electrodes. TiO_2 electrodes are made from a mixture of 0.2g of TiO_2 powder (Degussa, P25), 0.4 ml of nitrate solution (0.1 M), 0.08g of polyethylene glycol and a few drops of Triton X-100 pa. The mixture is made into pasta, then it is used to coat the SnO_2 conductive glass and then heated at a temperature of 450 °C for 2 hours.

Preparation of electrochromic materials from coloring plant. A total of 10g of material from beets, strawberries, and purple cabbage were extracted with 100ml of ethanol for 30 minutes. The anthocyanin concentration was measured with differential pH method according to Giusti and Worlstat (2001).[19]

Test of Electrochromic Materials as Intercalating Agents. Testing is done by adding anthocyanin extract to a solution of double stranded DNA isolation that was obtained from the Department of Anatomical Pathology FKUI on several variations of concentration. Observations on the ability of anthocyanin extract as an intercalating agent were done by UV-Vis spectrophotometer. The observed data are the peak absorption intensity changes and shifts in the peak

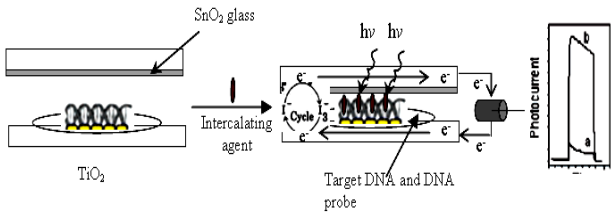


Figure 1. Photolectrochemical System to Measure DNA Concentration

absorption of DNA solution spectrum after the addition of anthocyanin extracts.

Biosensor preparation, hybridization, and detection. The photoelectrochemical system that was used in this research was 'sandwich' electrode that consisted of TiO_2 as a working electrode and SnO_2 as a counter electrode. The electrolyte that was used was Iodide-Iodine (I_2-KI) solution. The test was carried out at a DNA double helix so that an additional DNA probe was not needed (without hybridization steps). The data analyzed were a variation of current that was produced by the system to change DNA concentration. The photoelectrochemical system can be seen in Figure 1.

The DNA concentration was measured based on photoelectrochemical according to Teles et al.[20] The synthetic dengue-related oligonucleotide sequence 5'-GGT TAG AGG AGA CCC CTC CC-3' (target) and its complimentary counterpart, 5'-GGG AGG GGT CTC CTC TAA CC-3' (probe), were purchased as a lyophilized powder from Invitrogen. The TiO_2 electrode was then immersed in 10 µl of 4.00 nM DNA-probe solution in 50 mM TE buffer pH=7 for 1 hour. After being rinsed with 1 ml of TE buffer pH=7 to remove unbound DNA, the electrode surface was immersed in the TE buffer until further use.

The DNA-probe modified electrode was immersed in hybridization buffer (50mM of TE buffer pH=7) containing 0.75-3.00nM target sequence. The set was incubated at 60 ºC for 1 hour so that hybridization could occur. The electrode was finally washed with 1ml of TE buffer pH=7 to remove adsorbed (unhybridized) DNA-target.

Anthocyanin was attached to the hybridized target oligonucleotide intercalation after 20 minutes of incubation at 60 °C with a 10µL aliquot of 10µg/mL anthocyanin extract in TE buffer.

Photocurrent was measured at a variation of voltage and light intensity. The data analyzed were a variation of currents produced by the system to change DNA concentration.

Results and Discussion

Test of Electrochromic Materials as Intercalating Agents. Examinee materials such as ethidiumbromide (EtBr) and anthocyanin extracts were tested as intercalating agents. In this research, anthocyanin was obtained by alcohol extract from chromatic fruits such as beet, grape, purple cabbage, and strawberry. The absorption spectra of anthocyanin extract from strawberry can be seen on Figure 2.

On Figure 2, it can be seen that colour substance in strawberry can function as an intercalating agent. This condition can be perceived from the hypochromism effect and red-shift. The hypochromism effect emerges because some colour substances intercalate DNA molecule so that they degrade its absorbance value. As shown in Figure 2, the addition of DNA to anthocyanin extract resulted in an ~40% decrease and a ~2 nm red-shift of anthocyanin extract absorption band at 534 nm. This condition conforms to Zhiqiang et al. (2005)'s finding in which they discovered the intercalating effect of $Ru(bpy)_2^{2+}$ which derived from DNA molecule.[11]

Test of Photoelectrochemical System. The photoelectrochemical system was operated using anthocyanin extract from strawberry as an electrochromic agent. The anthocyanin extract from strawberry was selected because it cannot be chelated Ti^{+4} in the absence of carbonyl group that adjacent to hydroxyl group on one of benzene ring that chelating the ion.[18] It is expected that there is a significant difference in the response of current between systems with or without double-stranded DNA after the addition of anthocyanin extract. Thus, the anthocyanin dye will only bind to double -stranded DNA molecules that have been immobilized on TiO_2 and not bound to TiO_2 molecules.

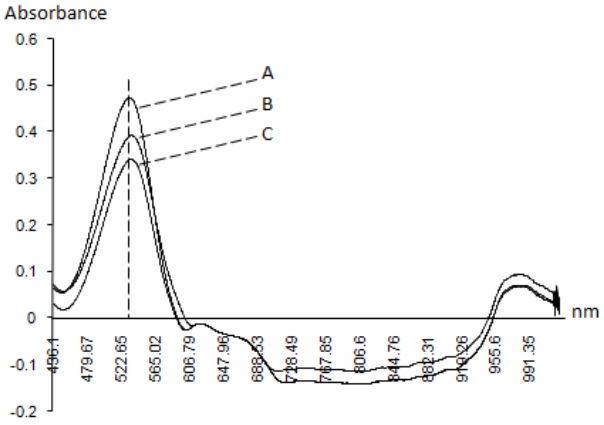
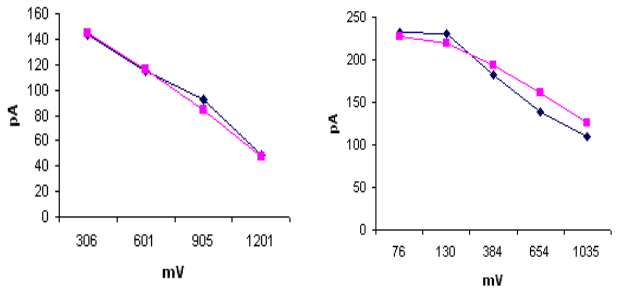


Figure 2. UV-Vis Absorption Spectra of Anthocyanin Extract as a Function to Increase DNA Concentration for Strawberry Extract (A), Strawberry Extract +0.2ppm DNA (B), Strawberry Extract +0.4ppm DNA (C)

The change of photocurrent against the external voltage applied to the photoelectrochemical system can be seen in Figure 3. Figure 3 shows that initially the external voltage will increase the current. However, at some point, the addition of greater external voltage (to positive electrode) will decrease the current. This condition is called reverse bias, or a condition that occurs in PN type semiconductor when P is associated with the negative pole of the battery and N is associated with the positive pole. In this case, the minority carriers (electrons) in P are moved to achieve the positive pole of the batteries. Similarly, the minority charge carriers (holes) in N are also moved to reach the negative pole. Therefore, in these circumstances, there is also a reverse biased current output even in very small quantities. This current is often called the reverse saturation current or leakage current.[21] Thus, the greater the external voltage of a current in the opposite direction against the leakage, the smaller the current is produced by a semiconductor.

Figure 3 (a) shows that with the control or without the addition of double stranded DNA, the photocurrent is either subjected or not subjected to light. This means that the dye is not bound to DNA by the washing process. This condition indicates that TiO_2 electrode that is sensitized with electrochromic substance will deliver more electron compared to electrode that is not sensitized by electrochromic substance.

In the reverse bias when the temperature of PN juction is raised, leakage current also increases. It means that the provision of energy will develop minority charge carriers in the PN junction. This effect is called photoconductive. In this situation, if light intensity subjected to photodiode is enlarged, the leakage currents will also increase. If the battery in the reverse bias circuit is removed and replaced with a load of resistor, the provision of light will lead to carriers of both holes and electrons. If light intensity is increased, the current output also becomes greater. This effect is called photovoltaic.[21]



(a) (b)

Figure 3. The Change of Current Produced by Photoelectrochemical System to Detect Double Stranded DNA for with Light (■), without Light (♦); (a) System without DNA Addition and (b) System with DNA Addition. The DNA Concentration was 10 ng/mL and the Anthocyanin Extract Concentration was 10 mg/mL. The Light Source was Bulb Lamp (25 watt, 535 Luxes at 25 cm)

Photovoltaic that indicates that the photoelectrochemical system is working can be seen in Figure 4. Figure 4 shows that the photocurrent increases in proportion to the increase of light intensity (lamp voltage). It shows that photoelectrochemical system is built between the double stranded DNA with anthocyanin- sensitized TiO_2 electrodes having photovoltaic effect.

Photoelectrochemical detection of Dengue-related oligonucletide concentration. Photocurrent changes in response to changes of the target DNA concentration can be seen in Figure 5. As shown in the figure, it can be concluded that the photoelectrochemical system can detect oligonucleotide sequences that are bound to the

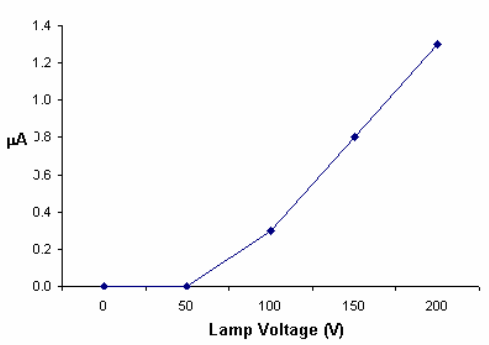


Figure 4. The Relationship Between Lamp Voltage and Photocurrents in the Photoelectrochemical System. Lamp Voltage is Proportional to Light Intensity. The System Used a 25-watt Halogen Lamp. The Light Intensity was 6000 Lux at 150 V. The DNA Concentration was 4.00 nM and the Concentration of Anthocyanin was 10 µg/mL

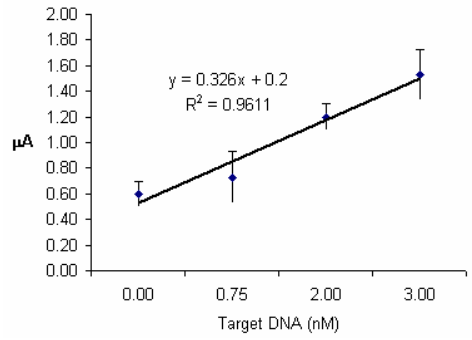


Figure 5. The Change of Current at the System Pertaining to the Change of Target DNA Concentration. The Light Source Used was a 25 watt Halogen Lamp (6000 Luxes at a Distance of 25 cms). The Concentration of DNA Probe was 4.00 nM, and the Concentration of Anthocyanin Extract was 10 µg/mL

DNA probe. The improvement of target DNA can be seen with the increase of electrical current produced by the increase in DNA concentration. Linearity (R2) is generated from the change of current in response to concentration changes of target DNA (in the concentration range of 0.75–3.00 nM), which was 0.9611.

Conclusion

Photoelectrochemical system using TiO_2 electrode and anthocyanin extract as electrochromic materials and DNA intercalation can detect the change of oligonucletide concentration. Linearity (R2) generated from the change of current in response to concentration changes of target DNA (in the concentration range of 0.75–3.00 nM) is 0.9611. Nevertheless, measurement of DNA concentration is needed using RT-PCR (real time Polymerase Chain Reaction) as a gold standard to measure sensitivity and specifity of the method.

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