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*Research Article*

# Label-Free Dengue Detection Utilizing PNA/DNA Hybridization Based on the Aggregation Process of Unmodified Gold Nanoparticles

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A label-free optical detection method based on PNA/DNA hybridization using unmodified gold nanoparticles (AuNPs) for dengue virus detection has been successfully developed. In this study, no immobilization method is involved and the hybridization of PNA/DNA occurs directly in solution. Unmodified AuNPs undergo immediate aggregation in the presence of neutral charge peptide nucleic acid (PNA) due to the coating of PNA on AuNPs surface. However, in the presence of complementary targets DNA, the hybridization of PNA probe with target DNA forms negatively charged complexes due to the negatively charged phosphate backbone of the target DNA. The negatively charged complexes adsorbed onto the AuNPs surface ensure sufficient charge repulsion, need for AuNPs dispersion, and stability in solution. The detection procedure is a naked eye method based on immediate color changes and also through UV-vis adsorption spectra. The selectivity of the proposed method was studied successfully by single base mismatch and noncomplementary target DNA.

## 1. Introduction

Dengue virus is an infectious tropical disease that poses a serious problem in the world [1]. The symptoms for dengue fever and dengue hemorrhagic fever are typical and can be seen after more than a week [2, 3]. There are no vaccines or drugs available to prevent or cure the disease caused by the dengue virus effectively [4]. To date, there is no specific therapeutic treatment for dengue virus infection. Prevention of the disease only focused on mosquito eradication strategies, which were reported after several cases in some areas with limited success [5].

It is crucial for a physician to diagnose dengue fever rapidly, properly, and accurately to achieve shorter operation time and to avoid excessive labor. Diagnosis based on existing symptoms is very problematic since the initial dengue virus infection symptoms are similar to those of influenza, measles, malaria, typhus, yellow fever, and other virus infections [5]. There are more conventional methods have been used for the detection of dengue virus. One of the most common methods is ELISA assays based on the detection of IgG and IgM antibodies to dengue virus [6]. However, the results are affected by cross-reactivity with other flaviviruses and require five days or more after infection to get the result. This is due to the lack of sufficient immune to produce detectable antibodies in patient's blood. Tissue culture and immunofluorescence are other conventional approaches for the detection of dengue virus [7–9]. Unfortunately, these two methods are limited in terms of specificity, sensitivity, ease of use, and speed.

Table 1: Sequences of oligonucleotides for PNA probe and DNA targets.

| Name | Sequences |
|---|---|
| Probe PNA | N′-TCG AGC AAG CCG TGC TGC CTG TAG CTC-C′ |
| Complementary target DNA | 5′-GAG CTA CAG GCA GCA CGG CTT GCT CGA-3′ |
| Noncomplementary target DNA | 5′-TGT AGC TCT ATG TTG AAT AGG AAG ATC-3′ |
| Single base mismatch target DNA | 5′-GAG CTA CAG GCA GCA CAG CTT GCT CGA-3′ |

Many researchers have used molecular assays based on nucleic acid amplification for dengue virus detections. In this method, firstly, dengue genomic RNA needs to be converted into DNA [10–14] using polymerase chain reaction (PCR). Double stranded DNA, which the product yields from PCR reaction, must be denatured before being used as probe hybridization based detection method. This method is not labor-free since it requires a molecular biologist to handle this assay. Additionally, PCR requires thermal cyclic instrumentation which is not cost effective as it involves high cost instruments and it is hard for miniaturization. Real-time polymerase chain reaction (RTPCR) [15], DNA microarrays (gene chip) [16], surface plasmon resonance biacore instrument [17], and GeneXpert system [18] offer fast and sensitive tools to detect the dengue virus disease. However, these instruments involve high cost and require well-trained employees for running. More efforts have been made to seek an ideal tool for fast, sensitive, low-cost, and easy-to-use dengue virus detection based on nucleic acids [19, 20]. The sensitivity of the detection was further enhanced by the use of nanomaterials without labels. Diagnosis of dengue virus using nucleic acid is more important. Various strategies have been developed to detect DNA sequences of dengue virus such as conventional bioassay [21, 22]. However, these conventional methods fail to provide enough specific information and require labeling with external reagents such as enzymes and/or fluorescent dyes. Additionally, the labeling procedure may cause suppression in the specific recognition of DNA-DNA hybridizations. Furthermore, the labeling procedure is time consuming and causes a high background signal. Label-free detection systems have become increasingly popular nowadays where it offers high possibility of realizing more convenient bioassay systems compared to conventional ones.

In this study, the development of a label-free method for detection of dengue virus target DNA sequence has been designed. The method is based on previous reported work [23] with slight modification. This colorimetric assay was successfully employed for the dengue DNA detection based on the aggregation of unmodified metallic nanoparticles. Peptide nucleic acid (PNA) molecules consist of neutral charge acts as a hybridization probe assay system used as a "coagulant" of citrate anion-coated AuNPs. Free PNA molecules induce particle aggregation in the absence of DNA because of the removal of charge repulsion due to the PNA coating on AuNPs. However, in the presence of target DNA, a complex of PNA-DNA forms, and the particles remain stable due to the negative charges of the DNA strands in the complexes adsorbed on the particle surface and repulsion process.

In this report, we have successfully designed colorimetric target DNA sequences for the determination of dengue virus. We also studied the specificity of proposed method via testing using noncomplementary target DNA. The similar experimental procedure was repeated with single-base-mismatch DNA for approving the good selectivity.

## 2. Materials and Methods

*2.1. Chemicals.* PNA and DNA were synthesized by Panagene Co. in Korea and First BASE Laboratories Sdn. Bhd. in Malaysia, respectively. Table 1 shows the sequences of PNA probes and the complementary, noncomplementary, and single base mismatch DNA targets. PNA is the probe that captures 27-mer complementary target DNA. The DNA target is from serotype I, dengue virus.

AuNPs was purchased from Cybeles (Malaysia) Sdn. Bhd with the size of 40 nm and was directly used without further purification and any dilution. Sodium dihydrogen phosphate ($NaH_2PO_4$) and disodium hydrogen phosphate ($Na_2HPO_4$) were purchased from Sigma-Aldrich and used without any purification for preparation of 10 mM phosphate buffer (PBS), pH 7. Sodium chloride (NaCl) and ethylenediaminetetraacetic acid (EDTA) were also supplied from Sigma-Aldrich and used without further purification.

*2.2. Preparation of Oligonucleotides.* PNA probes and DNA targets were diluted using 10 mM PBS pH 7 containing 100 mM NaCl and 0.1 mM EDTA to prepare a stock of 100 $\mu$M for each oligonucleotide.

*2.3. Procedure for DNA Detection.* The detection method for dengue virus was according to Kanjanawarut and Su, 2009 [23], with slight modifications. Previously, the assay was conducted using 150 $\mu$L of AuNPs with 13.2 nm average in size and with final PNA concentration of 1.0 $\mu$M. In this work, 140 $\mu$L of AuNPs was added into 96-well plates. Then, the hybridization of PNA probe and DNA target was done separately using different ratios of PNA/DNA (Table 2) with incubation time of 5 min. Then, 20 $\mu$L of PNA/DNA mixture was added into 96-well plates containing 140 $\mu$L of AuNPs solution. Absorption spectra were recorded after 10 min incubation at room temperature in the wavelength range of 400 to 800 nm. The same procedure was used for

Table 2: Experimental conditions for detection of specific DNA sequence, noncomplementary DNA, and single base mismatch based on the AuNPs aggregation process.

| Assay | AuNPs ($\mu$L) | PNA ($\mu$L) | Complementary DNA ($\mu$L) | Noncomplementary DNA ($\mu$L) | Single base mismatch DNA ($\mu$L) | Ratio ($V:V$) |
|---|---|---|---|---|---|---|
| A | 160 | — | — | — | — | — |
| B | 150 | 10 | — | — | — | — |
| C | 140 | 10 | 10 | — | — | 1:1 |
| D | 140 | 10 | — | 10 | — | 1:1 |
| E | 140 | 10 | — | — | 10 | 1:1 |

detection of noncomplementary and single base mismatch target DNA, as described previously.

## 3. Results and Discussion

*3.1. Determination of Specific Dengue Virus Target DNA.* The colorimetric assay for dengue virus detection using hybridization process of PNA/DNA has been conducted using free solution technique. The principle was based on aggregation of AuNPs in the presence of PNA. The aggregation was retarded in the presence of a target DNA to form PNA-DNA complex. However, for a noncomplementary DNA that forms PNA/DNA noncomplementary mixture, the aggregation of AuNPs was observed. In the presence of PNA, the red color of AuNPs solution turns into dark purple and the specific surface plasmon peak at wavelength 520 nm shifted to 680 nm. However, in the presence of target DNA, due to the hybridization of PNA with DNA (PNA/DNA complementary complex), no aggregation occurred and the solution remains in red color. This concept was proven using PNA probe and target DNA sequence specific for dengue virus from Serotype I.

Figure 1 shows the image and absorption spectrum for detection of specific dengue virus DNA sequence. Assay condition for the detection of specific dengue virus DNA sequence using unmodified AuNPs has been shown in Table 2. Final concentration of PNA and DNA solutions in AuNPs was 12 $\mu$M and 0.12 $\mu$M, respectively. The citrate-capped colloidal AuNPs typically have a specific surface plasmon peak at wavelength 520 nm, resulting in a red-colored solution as shown in Figure 1(A). In the absence of a complementary target DNA, free PNA probe sequence induced aggregation of AuNPs resulting in color shifted from red to purple-violet and observed that the absorption peak shifted from wavelength 520 nm to 680 nm; see Figure 1(B). In contrast, hybridization of PNA probe and complementary dengue virus DNA form a PNA/DNA complex without any change in red color of dispersed colloidal AuNPs as can be seen in Figure 1(C). This is due to the no aggregation process in the presence of PNA/DNA complementary complex. In the presence of a mixture of unhybridized PNA and noncomplementary dengue virus DNA, the red color of AuNPs solutions was changed to purple due to the aggregation process and appearance of the plasmon peak at 690 nm (Figure 1(D)). Theoretically, single-stranded DNA is able to stabilize AuNPs because DNA structure allows the nucleosides to interact with AuNPs effectively. In this case, the lack of single stranded noncomplementary DNA ability to protect AuNPs in the presence of PNA indicates that PNA molecule binds mostly to AuNPs. Interaction between DNA-AuNPs is less efficient due to the charge repulsion between the single-stranded noncomplementary DNA and the citrate anions on the particle surface that renders the interaction between PNA-AuNPs. Another study was further done to distinguish single base mismatch in the complementary targets (Figure 1(E)). The surface plasmon peak was observed at wavelength around 650 nm and the color changed from red to purple due to the aggregation process of free PNA molecules and AuNPs without addition of NaCl (5 M) as previously reported by Kanjanawarut and Su [23]. In the presence of single base mismatch, PNA-DNA hybridization efficiency was reduced, leaving some PNA and single base DNA target unhybridized. The free PNA molecules form an aggregation with nanoparticles, resulting in an increasing of absorbance at longer wavelengths. It was found that changes in visible absorption were observed due to aggregation of AuNPs prior to the addition of unhybridized PNA and single base mismatch DNA by shifting in plasmon peak to 650 nm.

Table 3: Ratio concentrations of PNA/DNA in the hybridization process. All the assay conditions were in the presence of AuNPs solution. (Assay 1 for AuNPs, 2 for AuNPs/PNA, and 3 to 8 for AuNPs with the complex of PNA/DNA, with different ratios.)

| Assay | PNA probe ($\mu$M) | Complementary DNA ($\mu$M) | Ratio (PNA : DNA) |
|---|---|---|---|
| 1 | — | — | — |
| 2 | 12 | — | — |
| 3 | 12 | 0.12 | 1 : 0.01 |
| 4 | 12 | 0.36 | 1 : 0.03 |
| 5 | 12 | 0.6 | 1 : 0.05 |
| 6 | 12 | 1.2 | 1 : 0.1 |
| 7 | 12 | 6 | 1 : 0.5 |
| 8 | 12 | 12 | 1 : 1 |

The sensitivity of the proposed colorimetric assay method based on unmodified AuNPs for quantifying concentration of dengue virus DNA target was conducted through the hybridization of 12 $\mu$M of PNA probe solutions with different complementary DNA target concentration at ratios of 0, 0.01, 0.03, 0.05, 0.1, 0.5, and 1, respectively (Table 3). The hybridization process of PNA/DNA was carried out based on

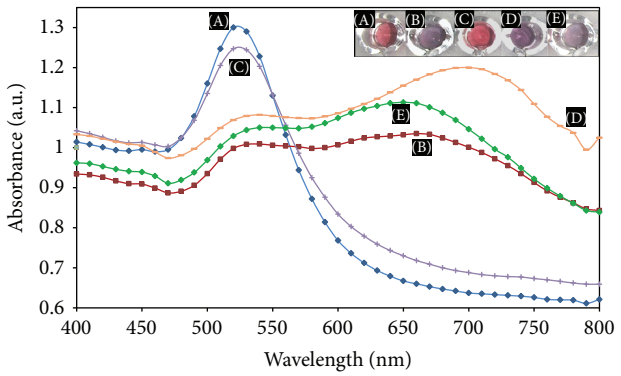


Figure 1: Detection of a specific dengue virus target DNA sequence using AuNPs. Adsorption spectra of (A) AuNPs, and AuNPs solution in the presence of (B) PNA probe, 27-mer, (C) complex of PNA and complementary DNA, (D) mixture of PNA and noncomplementary DNA, and (E) mixture of PNA and single base mismatch DNA. Insert is the photograph image of the color changes of assay conditions (A)–(E).

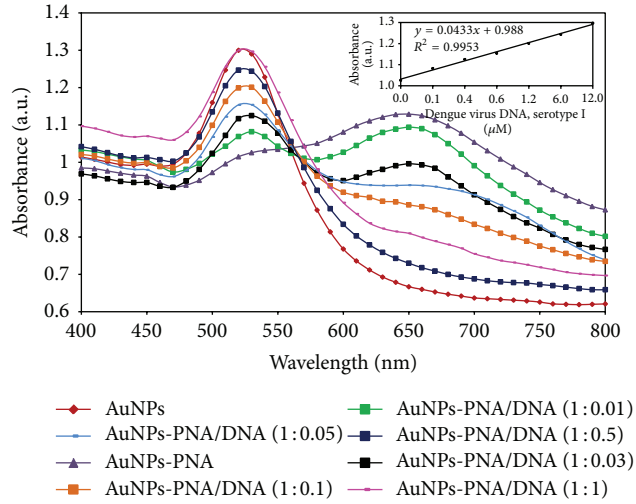


Figure 2: Absorption spectra for the sensitivity of colorimetric assay based on the unmodified AuNPs in the presence of 12 $\mu$M PNA probe. The corresponding spectra are referring to the 1 : 0.1, 1 : 0.5, and 1 : 1, respectively. Red line is the spectra for AuNPs as reference. Insert is the calibration graph for dengue virus DNA target at different concentrations.

conditions explained in Table 3 prior to addition into the 96-well plate containing AuNPs solutions.

The effect of dengue virus DNA target concentration was further explored by studying the changes in absorbance intensity at wavelength of 520 nm. It was found that the intensity at 520 nm was proportional to the dengue virus DNA target concentration directly. This is due to the presence of PNA-DNA complementary target complex that capped the AuNPs resulting in more charge repulsion in AuNPs solution. Figure 2 shows the absorption spectra of the hybridization of PNA/DNA complementary complex that increased gradually at wavelength of 520 nm with the increasing of dengue virus DNA target concentration. In the presence of PNA/DNA target concentration, the amount of dispersed nanoparticles increase and result in the gradually increment of absorbance at 520 nm. The changes in the absorbance can be seen for the DNA target concentration <0.12 $\mu$M with the PNA : DNA ratio of 1 : 0.01 compared to PNA alone. At the DNA target concentration of 0.12 $\mu$M, the second peak appeared at 650 nm and was largely distinguishable from PNA alone. The second peak of free PNA that occurred at 650 nm was referred to the aggregation of nanoparticles.

The increment in absorbance was proportional to the dengue virus DNA targets at 520 nm in the range of 0.0 to 12.0 $\mu$M as can be seen in Figure 2 (insert). The detection limit for the proposed colorimetric method was found to be 0.12 $\mu$M at PNA : DNA ratio of 1 : 0.01.

## 4. Conclusion

In this study, we demonstrated a label-free colorimetric method using unmodified AuNPs for the detection of specific dengue virus DNA target from Serotype I. PNA with neutral charge backbone was employed as a probe. PNA probe was designed specifically for the detection of dengue virus DNA target (Serotype I). In this method, the color changes are visualized by naked eye and also could be determined spectrophotometrically. AuNPs appeared in red color solution, and in the absence of DNA target solution PNA induces particle aggregation and changes the red color solution of AuNPs to purple and resulted in wavelength shifted from 520 nm to 650 nm. However, hybridization of PNA probe with DNA target form PNA-DNA complex adduct and the red color of AuNPs was stable due the negative charge of PNA-DNA complementary complex that ensures sufficient charge repulsion of the AuNPs to remain dispersed. This method is robust compared to the methods using DNA as probe because PNA is easier to handle compared to DNA. The selectivity of the proposed method was successfully studied for noncomplementary and single base mismatch target DNA.

## Conflict of Interests

The authors declare no conflict of interests.

## Acknowledgment

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