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# Fabrication of a Nanoparticle-based Sensor for the Detection of Dengue Virus-3 in *Aedes aegypti*

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**Abstract**

Dengue is considered as one of the major global health problems. Detection of the virus from mosquitoes located in an area will help warn the community and enable them to take immediate actions. A nanoparticle-based sensor was fabricated using DNAzyme-functionalized dextrin-capped gold nanoparticles to detect the presence of dengue virus serotype-3 (DENV-3) in *Aedes aegypti*. It utilizes the ribonucleic acid (RNA) cleaving ability of DNAzymes together with the visual detection of the aggregation of gold nanoparticles. Successful detection of DENV-3 was performed using synthetic DENV-3 target oligonucleotide and extracted RNA from *A. aegypti*.

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The nanoparticle-based sensor was found to be serotype-specific for DENV-3 by demonstrating a positive result only with DENV-3. This was performed using extracted RNA of different dengue serotypes from *A. aegypti*. In this study, the fabricated nanoparticle-based sensor can detect target concentration for as low as 0.1 μM using synthetic DENV-3 target and 5 x $10^2$ PFU/mL using extracted RNA from *A. aegypti*.

The nanobiosensor presented in this study provides a simple, faster, "greener", and portable way of detecting the DENV-3 in mosquitoes for epidemiological purposes.

***Keywords:*** colorimetric; dengue; DNAzymes; gold nanoparticles; nanobiosensor.

**1. Introduction**

The dengue virus (DENV), member of the Flaviviridae family, is a major health threat in tropical and subtropical areas around the world because of the high number of cases and deaths due to dengue [1]. *Aedes aegypti* and *Aedes albopictus* are the common carriers of the dengue virus. A mosquito becomes infected with the virus by biting an infected person. It can later transmit the virus by biting a healthy person. Mosquitoes are the ones responsible for the transmittance of dengue since the infection cannot spread directly from one person to another [2].

Dengue fever is a flu-like illness which may occasionally develop into a potentially lethal complication called severe dengue that can affect infants, young children and adults. Almost 40% of the world's population is at risk of this infection. Symptoms include severe headache, pain behind the eyes, muscle and joint pains, nausea, vomiting and swollen glands or rash which usually last for 2-7days. These symptoms manifest 4-10 days after being bitten by an infected mosquito [3].

In 2013, around 117,658 dengue cases were reported in the Philippines during the first nine months with 433 recorded deaths. At least 40% of the infected patients were children aged 1-10 years old [4]. Prevention from mosquito bites is given priority in order to help minimize the number of dengue cases.

There are four dengue serotypes that can cause dengue infection: DENV-1, DENV-2, DENV-3, and DENV-4. One of the most common dengue serotype in the Philippines is DENV-3 [5]. Recovery from an infection caused by one serotype does not guarantee permanent immunity against infections from the other serotypes. It was also reported by Dr. Scott Halstead in the 1960s that people who were exposed to a second dengue infection have a greater risk of developing severe dengue compared to those who had not been infected before [6].

Dengue virus detection is usually done for diagnosing dengue infections in patients after the onset of their symptoms but due to the increased effort of preventing dengue infections, dengue virus detection has also been applied in mosquitoes [7-8]. By detecting the presence of dengue-carrying mosquitoes in an area, necessary measures may be taken to prevent future infections. However, current methods for this type of detection cannot be used on site in local communities where rapid, sensitive and low-tech detection methods are most needed [9] due to certain limitations. These methods may either be too expensive, not sensitive enough, labor-intensive or time-consuming [10].

Nanoparticle-based sensors have been widely used in pathogen detection. They were reported to provide a rapid and sensitive detection of different pathogens such as Human Immunodeficiency Virus Type 1 (HIV-1) p24 antigen [11], *Salmonella* DT104 [12], and *Escherichia coli* O157:H7 [13] among others. Gold nanoparticles (AuNPs), in particular, show promising applications in biomedicine as a tool in biosensing.

This study aims to exploit the properties of the synthesized dextrin-capped gold nanoparticles to fabricate a nanoparticle-based sensor and provide a rapid, cost-effective, "greener", and portable way of detecting the dengue virus-3 in *Aedes aegypti*. The specific objectives of the study are to conjugate the DNAzyme detector probes to the synthesized dextrin-capped gold nanoparticles; to determine the optimum parameters for the colorimetric detection using DNAzyme-functionalized gold nanoparticles; to perform the colorimetric detection using both synthetic DENV-3 target oligonucleotide and extracted RNA from *Aedes aegypti*; to assess the selectivity of the nanoparticle-based sensor; and to determine the effect of varying target concentration on the absorbance of the aggregated gold nanoparticles.

## 2. Materials and Methods

### *2.1 Materials and Equipment*

Sodium chloride, sodium acetate, magnesium chloride, sodium phosphate (monobasic and dibasic), and glacial acetic acid were purchased from Ajax Finechem Pty Ltd. (Australia). Sodium dodecyl sulfate, dithiothreitol, and Trizma base were purchased from Sigma Aldrich (USA). All oligonucleotide sequences were ordered from Integrated DNA Technologies, Inc. (USA). Milli-Q Type 1 water and synthesized gold nanoparticles were obtained from the Nano-Biotechnology Lab, BIOTECH-UPLB.

Measurements of pH were conducted using Horiba pH meter F-17. Centrifugation was performed using Allegra X-30R Centrifuge and spectrophotometric measurements were obtained using Shimadzu UV-1800 Spectrophotometer. Statistical analyses were carried out using Statistical Analysis Software (SAS/STAT).

### *2.2 Selection of the Detector and Target Probes*

The sequence of the 10-23 DNAzyme that served as the detector probe for the sensor was based on the study of Carter et al. [9]. It was designed to bind specifically to a region unique to the DENV-3 genome in comparison with the other dengue serotypes. The strain and Genbank GenInfo identifier used for DENV-3 was DENV type 3 strain ThD3 0010 87 (strain H87). The sequence of the detector and target probes are shown in Table 1.

**Table 1:** Nucleotide sequences of the detector and target probes.

| Probe | Sequence |
|---|---|
| Detector Probe | 5'-TA GCC AAG AGG CTA GCT ACA ACG ATC CTG CTG T-3' |
| Target Probe | 5'-TTC CAC CGA CAG CAG GAG TCT TGG CTA GAT GGA CT-3' |

The detector probe is the 10-23 DNAzyme capable of cleaving RNA molecules upon binding. The binding arms

consist of 9 base pairs while the catalytic core consists of 15 base pairs. The target probe contains the complementary sequence of the binding arms of the detector probes. Synthetic DNA oligonucleotide was used as the target probe while the DNAzyme detector probe was thiol-modified at the 3'-end for the functionalization of the gold nanoparticles.

### *2.3 Extraction of RNA from Aedes aegypti*

The extracted RNA from dengue and non-dengue containing *Aedes aegypti* were obtained from the Medical Entomology Department of the Research Institute for Tropical Medicine (Muntinlupa, PH). Five (5) mosquitoes were pooled in a 1.5-mL microcentrifuge tube where 500 μL PBS was added as the grinding medium. The concentration of the stock viruses are as follows: DENV-1 ($1.25 \times 10^5$ PFU/mL); DENV-2 ($2.5 \times 10^5$ PFU/mL); DENV-3 ($5.0 \times 10^5$ PFU/mL); and DENV-4 ($0.5 \times 10^5$ PFU/mL).

### *2.4 Synthesis of Dextrin-capped Gold Nanoparticles*

The synthesized gold nanoparticle solutions were obtained from the Nano-Biotechnology Laboratory, BIOTECH-UPLB. It was generated using a one-step alkaline synthesis as described in the study of Anderson et al. [14]. Dextrin was used as the capping agent and sodium carbonate as the reducing agent for chloroauric acid. The absorbance peak of the dextrin-capped gold nanoparticles was observed at 520 nm. The average diameter of the nanoparticles were determined to be $6.82 \pm 1.22$ nm using Transmission Electron Microscopy (TEM) imaging. It has an average zeta potential of -26.8 mV indicating that the system is significantly stable. The synthesized particles were diluted (1:10 dilution) with Milli-Q Type 1 water.

### *2.5 Functionalization of Synthesized Gold Nanoparticles with Thiolated DNAzymes*

The functionalization of the synthesized gold nanoparticles (AuNP) with DNAzymes was based on the study of Liu and Lu [15] with a few modifications. The thiolated DNAzyme was first activated using 0.2 M dithiothreitol (DTT) as described in a study by Zhang et al. [16]. In the experiment, 30 μL 100 μM thiolated DNAzyme (thiol-DNAzyme) was mixed with 30 μL 0.2 M DTT in 1.5-mL Eppendorf tubes. The tubes were then wrapped in aluminum foil and were allowed to stand for 2 h. Then, 50 μL 10 mM acetate buffer (pH 5.2) and 1.0 mL AuNP solution were added. The tubes were incubated at room temperature for 24 h. Ten (10) μL 500 mM Tris acetate buffer (pH 8.2) was added before adding 10 μL 1.0 M NaCl solution. The tubes were again incubated for 24 h. After the said time period, the tubes were centrifuged at 13,000 rpm at 28°C for 15 min using a refrigerated centrifuge to remove unreacted thiol-DNAzymes. The supernatant was removed and the nanoparticles were dispersed in 500 μL buffer containing 2.5 mM Tris acetate (pH 8.2), 10 mM NaCl, and 0.001% SDS. The tubes were again centrifuged at 13,000 rpm at 28°C for 15 min to remove the remaining unreacted thiol-DNAzymes. After removing the supernatant, the nanoparticles were resuspended in 300 μL buffer containing 30 mM NaCl and 2.5 mM Tris acetate (pH 8.2) and re-centrifuged at 13,000 rpm at 28°C for 15 min. The DNAzyme-functionalized gold nanoparticles (DNAzyme-AuNP) were finally dispersed in 200 μL buffer containing 2.5 mM Tris acetate, 10 mM NaCl, and 0.005% SDS.

### *2.6 Optimization of Parameters for the Colorimetric Detection of DENV-3*

The optimal amount of $MgCl_2$ for the colorimetric detection of DENV-3 was determined by mixing 50 µL DNAzyme- AuNP and varying volumes (0, 5, 10, and 15 uL) of 10 mM $MgCl_2$. These reaction mixtures were incubated at room temperature for 30 min before noting the final color of the solutions. Another batch of DNAzyme-AuNP were prepared and subjected to the same treatments. The absorbances of the mixtures were determined every 5 min for 30 min using a UV/Vis spectrophotometer. Then, the optimal amount of NaCl was determined by mixing 50 uL DNAzyme-AuNP with 5 µL 10 mM $MgCl_2$ and varied volumes (0, 5, 10, 15 and 20 µL) of 1.0 M NaCl. The optimum amount was determined qualitatively by noting the resulting color of the solutions after incubating the samples at room temperature for 30 min. Absorbances of the solutions at 520 nm were also determined. The experiment was done in triplicates.

### *2.7 Colorimetric Detection of DENV-3 using Functionalized DNAzyme-AuNP*

One hundred (100) µL DNAzyme-AuNP was mixed with 20 µL 100 µM target oligonucleotide, 10 µL 10 mM $MgCl_2$, and 10 µl 1.0 M NaCl to perform the detection. The resulting color of the mixtures was observed every 5 min for a span of 30 min. A color change from pink to purple indicates a positive result. The reaction mixture containing DNAzyme-AuNP with $MgCl_2$ and NaCl without the target RNA served as the blank while the reaction mixture containing DNAzyme-AuNP, $MgCl_2$ and NaCl with RNA extracted from dengue-free *Aedes aegypti* served as the negative control. The experiment was done in triplicates. The mixtures were further analyzed by determining their absorbance at 520 nm using a UV/Vis spectrophotometer.

### *2.8 Selectivity Analysis of the Colorimetric Detection of DENV-3*

To determine the specificity of the sensor, it was also tested against the other dengue serotypes. The extracted RNA of different dengue serotypes from *Aedes aegypti* obtained from the Research Institute for Tropical Medicine (RITM) was used in the analysis. The same procedure was followed for the colorimetric detection. The reaction mixture containing only DNAzyme-AuNP, $MgCl_2$, and NaCl without any extracted RNA served as the blank. The experiment was performed in duplicates at the Medical Entomology Laboratory of RITM (Muntinlupa City, Manila). The absorbances of the solutions were then determined using a UV/Vis spectrophotometer.

### *2.9 Effect of Varying Target Concentration on the Absorbance of Aggregated Gold Nanoparticles*

To determine the effects of varying target concentration on the absorbance of aggregated gold nanoparticles, the nanoparticle-based biosensor was tested against different concentrations of the DENV-3 target. Two setups were prepared. The first set-up using synthetic oligonucleotide was performed at the Nanobiotechnology Laboratory of BIOTECH (Los Baños, Laguna) while the second set-up utilizing extracted RNA from *Aedes aegypti* was performed at the Medical Entomology Laboratory of RITM (Muntinlupa City, Manila). For the synthetic target, the concentrations used were 100, 10, 1.0, 0.1, 0.01, and 0.001 µM. For the extracted RNA, the concentrations used were $5 \times 10^4$, $5 \times 10^3$, $5 \times 10^2$, $5 \times 10^1$, $5 \times 10^0$, $5 \times 10^{-1}$ PFU/mL. The experiment was done in duplicates. The absorbances were determined using a UV/Vis spectrophotometer.

## 3. Results

### *3.1 Principle of the Colorimetric Detection of DENV-3 using DNAzyme-AuNP*

The detection involves the binding of the DNAzyme-functionalized gold nanoparticles (DNAzyme-AuNP) to the target region of the DENV-3 genome. In the presence of $Mg^{2+}$ ions, the DNAzyme is activated and cleaves the RNA molecule. This activity causes the deshielding of gold nanoparticles from each other leading to aggregation in the presence of NaCl. This aggregation can be visually observed as a color change from pink to purple. The color change is the indication of a successful detection of DENV-3 that can be quantified using UV/Vis Spectrophotometry. For mixtures that showed a positive result, a significant decrease in the absorbance at 520 nm was observed in comparison with that of the pure DNAzyme-AuNP solution. If the target DENV-3 is not present in the mixture, no aggregation will occur for the cleavage activity is not triggered and the solution will remain pink. Also, if either $MgCl_2$ or NaCl is not present in the reaction mixture, no aggregation will also occur for they are needed to facilitate the cleavage activity of the DNAzyme and aggregation of gold nanoparticles, respectively.

The principle of this study was based on the study of Carter et al. [9] which reported a gold nanoparticle-based sensor mainly for DENV-2 using gold nanoparticles purchased from Sigma Aldrich (USA). This principle was applied for the detection of the most common dengue serotype in the country which is DENV-3 in *Aedes aegypti*. Synthesized dextrin-capped gold nanoparticles were used instead of purchasing pure gold colloidal solutions from other laboratories to provide a cheaper alternative for the sensor.

### *3.2 Functionalization of Synthesized Gold Nanoparticles with Thiolated DNAzymes*

Gold nanoparticles were conjugated to thiolated DNAzymes through the formation of partially covalent Au-S bonds. Since synthetic thiolated oligonucleotides, in general, are shipped in their oxidized form (the sulfur atoms are protected by an S-S bond), a reduction step is necessary before using the said oligonucleotides. In the experiment, instead of using Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) as indicated in the study of Liu and Lu [15], dithiothreitol (DTT) was used as performed in the study conducted by Zhang et al. [16].

The thiolated DNAzymes were first reduced using DTT. The general reaction is shown in Figure 6. DTT breaks the disulfide bonds leaving the thiol groups attached to the DNAzymes. The deprotected thiolated oligonucleotide can now undergo reactions such as conjugation reactions for the functionalization of gold nanoparticles.

Conjugation of the thiolated DNAzymes occur through the formation of 'partially covalent' Au-S bonds. The interaction is comparatively weaker than the bond between gold atoms but is still strong enough to prevent detachment [17]. Excess DNAzymes and other species were removed from the reaction mixture through centrifugation. The DNAzyme-functionalized AuNP were dispersed in a buffer containing 2.5 mM Tris acetate, 10 mM NaCl, and 0.005% SDS.

The whole functionalization step was done in three (3) days since there were necessary incubation periods of 24

h in some steps. The obtained functionalized gold nanoparticle solutions were pink in color as shown in Figure 2.

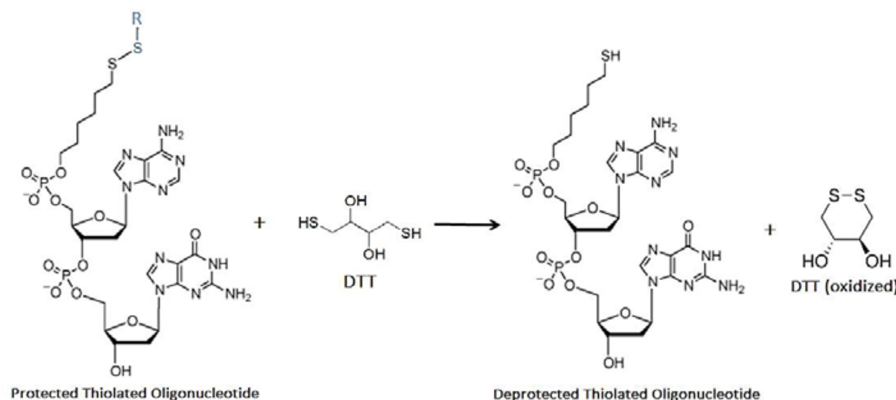


**Figure 1:** Reduction reaction of thiolated oligonucleotides using DTT.

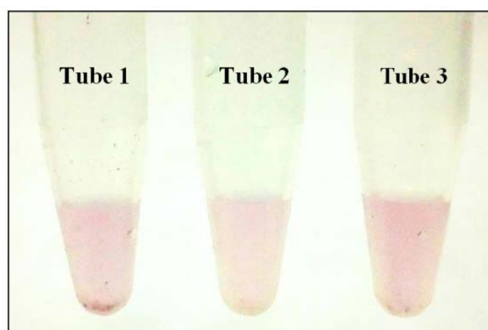


**Figure 2:** Functionalization of dextrin-capped gold nanoparticles with DNAzymes.

### *3.3 Optimization Parameters for the Colorimetric Detection of DENV-3*

As discussed previously, the colorimetric detection uses magnesium chloride ($MgCl_2$) and sodium chloride (NaCl) for the desired reaction to occur. However, some studies have reported that excess amounts of these compounds can cause undesired aggregation of the gold nanoparticles in the solution prior to the detection [18]. This is why it was necessary to first determine the optimal amounts of both compounds before conducting the detection with the target oligonucleotide.

The optimum amount of magnesium chloride was initially determined qualitatively by observing the resulting color of the solutions after addition of different amounts of $MgCl_2$. A color change of pink to purple indicates aggregation of the gold nanoparticles due to the instability of the system.

Based on the qualitative observations as seen in Figure 3, 50 μL DNAzyme-functionalized gold nanoparticle solutions containing more than 5 μL 10 mM $MgCl_2$ were already unstable after 30 min of incubation. This is evidenced by a color change of pink to purple which indicates that the gold nanoparticles have already aggregated. For the colorimetric detection, the optimum amount of $MgCl_2$ is the amount which is enough to

activate the DNAzymes but not too much that will initiate the premature aggregation of the gold nanoparticles. Thus, the optimum amount of 10 mM $MgCl_2$ was determined to be 5 µL based on the qualitative results.

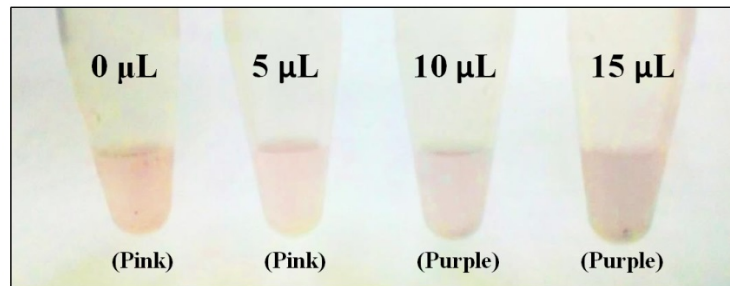


**Figure 3:** Qualitative determination of the optimum amount of $MgCl_2$ for the hybridization of DNAzyme-AuNP and the target DENV-3.

To further support the said observations, the absorbances of the mixtures were monitored every 5 min after the addition of appropriate amounts of $MgCl_2$. This was done by preparing another batch of samples with the same treatments. The absorbances of these samples were read using a UV-Vis spectrophotometer at time 0-30 min upon addition of appropriate amounts of 10 mM $MgCl_2$. The generated graph of the absorbances against time is given in Figure 4.

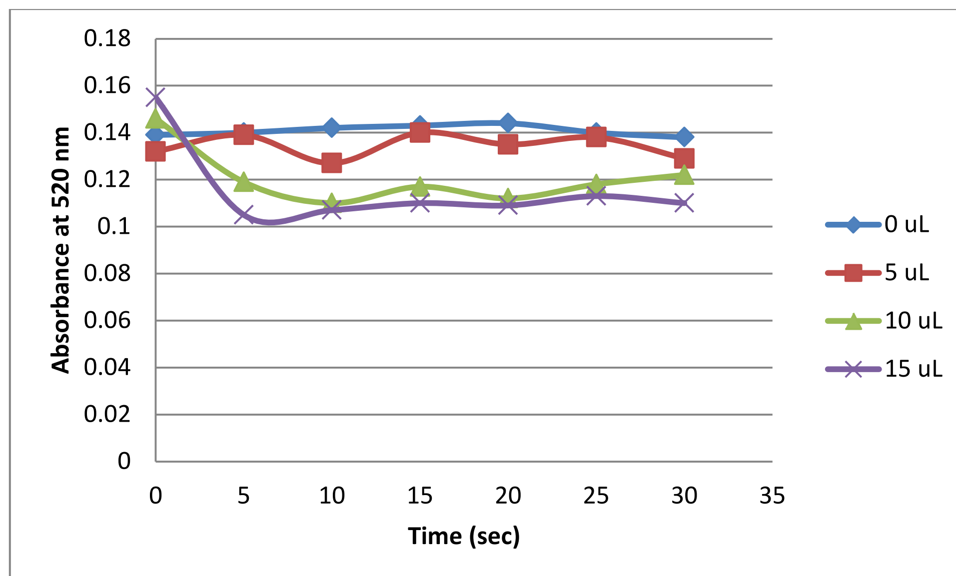


**Figure 4:** Quantitative determination of the optimum amount of $MgCl_2$ for the hybridization of DNAzyme-AuNP and the target DENV-3.

It was observed from the graph that after 5 min of reaction time, the absorbances of DNAzyme-AuNP solutions containing more than 5 µL of 10 mM $MgCl_2$ decreased and sustained a lower absorbance throughout the incubation period. This observation coincides with the qualitative results of the first batch of samples wherein solutions containing more than 5 µL showed deviations from the pure DNAzyme-AuNP solution. The decrease in the absorbance is accounted by the color change of the sample from pink to purple due to the aggregation of

the gold nanoparticles. At 5% by LSD, the absorbances of the mixtures containing more than 5 μL 10 mM $MgCl_2$ were found to be significantly different after 5 min of reaction time following the addition of appropriate amounts of $MgCl_2$. The observations coincides with what were reported in the study of Carter et al. [9] wherein there was a decrease in the absorbance of the mixtures that turned purple. Both the qualitative and quantitative results showed that the optimum amount of 10 mM $MgCl_2$ for the colorimetric detection is 5 μL.

For the optimum amount of NaCl, 50 μL DNAzyme-AuNP containing the optimum amount of $MgCl_2$ which is 5 μL was tested against different volumes of 1.0 M NaCl. This was determined qualitatively by noting the final color of the solutions after incubating the samples at room temperature for 30 min as seen in the Figure 5.

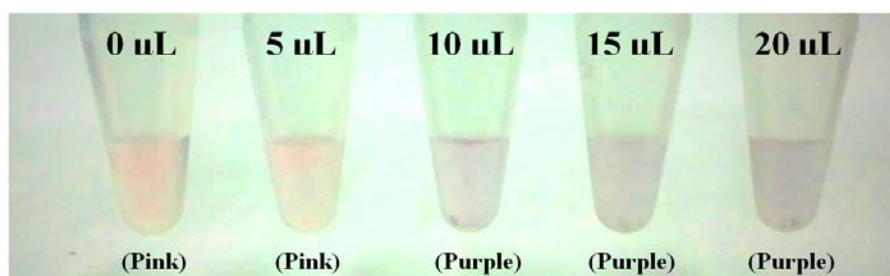


**Figure 5:** Qualitative determination of the optimum amount of 1.0 M NaCl for the hybridization of DNAzyme-AuNP and the target DENV-3.

Based on Figure 5, aggregation of the nanoparticles was observed at amounts higher than 5 μL 1.0 M NaCl. This indicates that at 5 μL, the mixture was still stable hence no aggregation was observed. The color of the mixture with 5 μL 1.0 M NaCl was still similar to that of the DNAzyme-AuNP solution without NaCl which means that there was no significant effect on the stability of DDZ-AuNP at this amount of NaCl. Qualitative results show that 5 μL is the optimum amount of 1.0 M NaCl for the hybridization of DNAzyme-AuNP with the DENV-3 target.

Quantitative measurements using a UV/Vis spectrophotometer was used to further analyze the results. After the incubation period of 30 min, the absorbances of the mixtures at 520 nm were determined. The obtained values are shown in Figure 6. Spectrophotometric measurements showed that the mixtures that aggregated after 30 min of incubation time based on the qualitative observations have lower absorbance values compared to the mixture containing DNAzyme-AuNP and $MgCl_2$ without NaCl. At 5% by LSD, the absorbances of these mixtures were significantly different from the absorbance value of the mixture without NaCl. This significant difference is accounted by the aggregation of the nanoparticles. The quantitative data supports the qualitative observations that 5 μL 1.0 M NaCl was the optimum amount for the colorimetric detection.

The optimum amounts of the components of the colorimetric detection method were determined to be 5 μL 10 mM $MgCl_2$ and 5 μL 1.0 M NaCl for every 50 μL DNAzyme-AuNP solution. Both experiments for the determination of the optimization parameters were conducted in triplicates.

### *3.4 Colorimetric Detection of DENV-3 using Functionalized DNAzyme-AuNP*

The detection was initially performed using 100 µL DNAzyme-AuNP with 20 µL 100 uM synthetic DENV-3 target oligonucleotide together with 10 µL 10 mM $MgCl_2$ and 10 µL 1.0 M NaCl. The reactions mixtures were then incubated at room temperature. The experiment was performed in triplicates. The tube that served as the blank contains all the components of the reaction mixture for the functionalization of the gold nanoparticles except the DNAzyme detector probe. The tube labeled "Synthetic Target" contains the functionalized DNAzyme-AuNP with the synthetic DENV-3 target oligonucleotide. The tube labeled with "Dengue-free Extracted RNA" contains the functionalized DNAzyme-AuNP with extracted RNA from dengue-free *Aedes aegypti*.

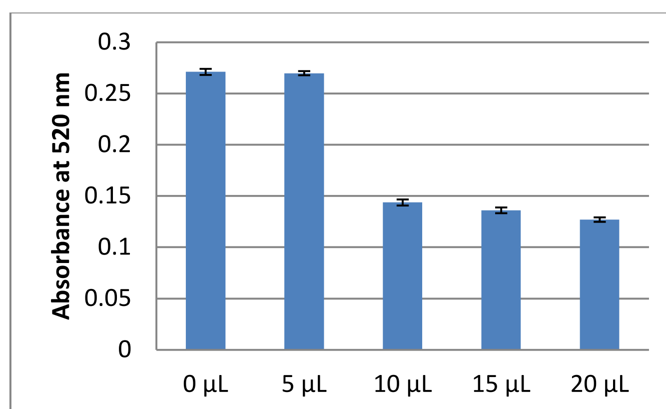


**Figure 6:** Quantitative determination of the optimum amount of NaCl for the colorimetric detection of DENV-3.

During the performance of the detection, the synthetic DENV-3 target, $MgCl_2$ and NaCl was also added to the blank. No significant color change was observed which shows that without the presence of the DNAzyme detector probes in the mixture, aggregation will not be possible. As early as five (5) min, a color change from pink to purple can be seen on the tube containing the synthetic DENV-3 target. This indicates aggregation of the gold nanoparticles which can only be triggered by the cleavage activity of the DNAzymes due to the presence of the DENV-3 target oligonucleotide in the mixture. Also, the negative control containing the extracted RNA from dengue-free *Aedes aegypti* remained pink indicating an expected negative result. This observation showed that only the presence of the DENV-3 target was able to induce the aggregation of the gold nanoparticles in the mixture. The results of the colorimetric detection are shown in Figure 7.

**Figure 7:** Colorimetric detection of DENV-3 using gold nanoparticles functionalized with DNAzymes.

The summary of the results for the colorimetric detection are tabulated in Table 2. From these results, the DNAzyme-AuNP based sensor was successful in detecting the presence of the synthetic DENV-3 target oligonucleotide containing the base sequence uniquely found in the DENV-3 genome. The results can be obtained for as early as five (5) min of reaction time and do not require any specialized equipment.

**Table 2:** Colorimetric detection of DENV-3 using DNAzyme-AuNP.

| Label | Description | Color | Result (+/-) |
|---|---|---|---|
| w/ Synthetic target | DDZ-AuNP with synthetic DENV-3 target | purple | + |
| w/ Dengue-free extracted RNA | DDZ-AuNP with extracted RNA from dengue-free *Aedes aegypti* | pink | - |

After the incubation period, the samples were diluted to 2 mL and were analyzed using UV/Vis Spectrophotometry to determine the absorbances of the mixtures at 520 nm. The absorbance values were compared with that of the pure DNAzyme-AuNP solution as seen in Figure 8. At 5% by LSD, there was a significant decrease in the absorbance of the mixture containing the synthetic DENV-3 target as compared to the blank which can be accounted by the observed color change of pink to purple. For the negative control, however, there was no significant difference observed on its absorbance compared with that of the blank.

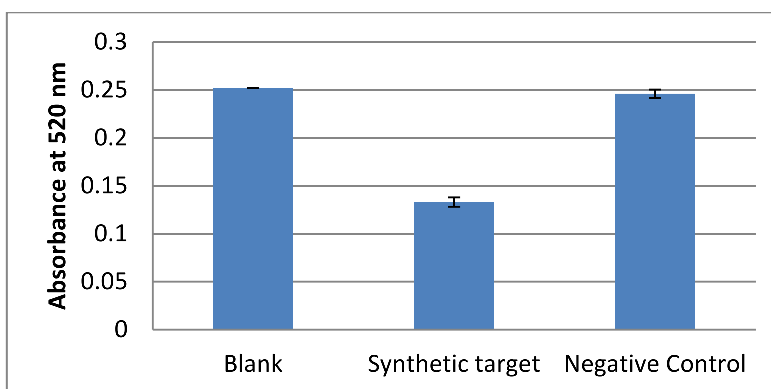


**Figure 8:** Quantitative analysis of the colorimetric detection of DENV-3 using UV/Vis spectrophotometry.

In cooperation with the Research Institute for Tropical Medicine (RITM), the DNAzyme-AuNP-based colorimetric detection method was performed on extracted RNA from *Aedes aegypti* carrying DENV-3. The said institute provided the extracted RNA used for the analysis as well as the facility for the performance of the experiment. The detection method was performed on three samples. Sample 1 contained RNA extracted from dengue-free *Aedes aegypti*. Sample 2 contained DENV-3 RNA extracted from *Aedes aegypti* using PBS as grinding medium (method 1) while sample 3 contained DENV-3 RNA extracted from *Aedes aegypti* using Trizol as grinding medium (method 2). Each type of extraction utilized five (5) mosquitoes. The concentration of the viruses obtained was 5 x $10^5$ PFU/mL. The extracted RNA from method 1 and the negative control were both colorless while the extracted RNA from method 2 was pink due to the pink color of Trizol.

The detection was performed by adding twenty microliters (20 µL) of the extracted RNA to each reaction tube containing 100 µL DDZ-AuNP before adding 10 µL 10 mM $MgCl_2$ and 10 µL 1.0 M NaCl. The results are shown in Figure 9.

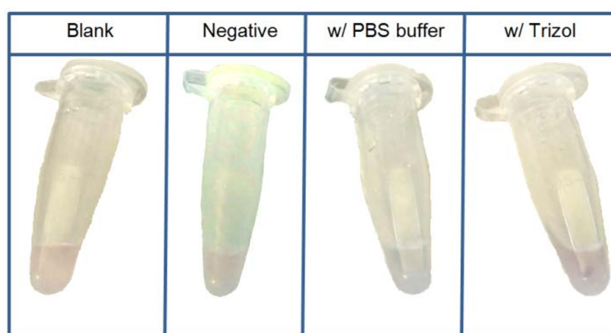


**Figure 9:** Colorimetric detection of DENV-3 using extracted RNA from *Aedes aegypti*.

Extraction of RNA from mosquitoes is commonly carried out by crushing mosquito tissues in a grinding medium to aid cell lysis and release the RNA in the mixture. PBS and Trizol are one of the frequently used grinding medium for the RNA extraction in RITM.

After performing the detection, the reaction mixtures containing the negative control remained pink indicating an expected negative result. For the samples containing the extracted RNA from method 1, the solutions turned purple indicating a positive result. This means that the DNAzyme-AuNP based sensor was successful in the detecting the presence of DENV-3 RNA extracted from *Aedes aegypti* using PBS as grinding medium.

For the samples containing extracted RNA using method 2, the solutions remained pink indicating a false negative result which can be due to the initial pink color of the extracted RNA caused by the presence of Trizol. This means that the results of the colorimetric detection using RNA extracted with Trizol cannot be reliable. Even if the nanoparticles have aggregated, it cannot be verified qualitatively since the pink color of Trizol masks the expected color change of pink to purple. The summary of the results are shown in Table 3.

**Table 3:** Summary of the colorimetric detection using RNA extracted from *Aedes aegypti*.

| Sample | Condition | Result |
|---|---|---|
| 1 | RNA extracted from dengue-free *Aedes aegypti* | (-) |
| 2 | RNA extracted using PBS | (+) |
| 3 | RNA extracted using Trizol | false (-) |

These results showed that in performing the colorimetric detection of DENV-3 using the nanoparticle-based sensor in this study, PBS is the preferred grinding medium to use. Unlike Trizol, PBS is colorless that is why the extracted RNA produced was also colorless. The use of PBS did not produce any observed unwanted effects on the results. For Trizol, however, the pink color of the reagent causes interference on the resulting color of the mixture upon addition of the extracted RNA and the other components of the sensor hence a false negative

result was obtained. The color of Trizol obscured the resulting color of the mixture that is why the color change due to the aggregation of the gold nanoparticles was not properly observed. These results show that the colorimetric detection using the DNAzyme-functionalized gold nanoparticles can be successfully performed on mosquitoes given that the appropriate grinding medium for the RNA extraction was used.

The most common method used for the detection of dengue in the country is RT-PCR. This method detects the presence of dengue viruses through the transcription of RNA into complementary DNA (cDNA) which is then amplified using primers with the use of a thermocycler. The products of the reaction are then analyzed through gel electrophoresis. The results of this method can be obtained as early as 1-2 days with around 20-25 samples per processing. With the fabricated nanoparticle-based sensor in this study, the results of the analysis can be obtained for as early as 5-10 min. It provides a more rapid way of detecting dengue viruses without needing the use of special equipment.

The sensor which is composed of the DNAzyme-AuNP solution, $MgCl_2$ and NaCl solutions can also be easily transported from one location to another without significantly affecting the quality of the results as long as the components are in a safe container and are not exposed to very high or very low temperatures. This is because at very high temperatures, degradation of the DNAzymes may occur while at very low temperatures, aggregation of the gold nanoparticles may occur despite being conjugated to DNAzymes.

The portability of the sensor was demonstrated in this study since the prepared solutions were transported from the Nano-Biotechnology Laboratory of BIOTECH (Los Baños) to the Medical Entomology Laboratory of RITM (Muntinlupa City, Manila) for the conductance of the colorimetric detection using extracted RNA from *Aedes aegypti*. The sensor was transported because the extracted RNA used in the experiment must be freshly prepared since degradation of RNA can easily occur if the sample is not stored immediately at very low temperatures or if not used instantly. These observations show that the fabricated nanoparticle-based sensor can provide a simple, portable, and faster way of the detecting dengue viruses in mosquitoes.

### *3.5 Selectivity Analysis of the Colorimetric Detection of DENV-3*

Aside from successfully detecting DENV-3, the specificity of the sensor for this dengue serotype was also determined. This was done by testing the DNAzyme-AuNP based sensor with the extracted RNA from other dengue serotypes. The extracted RNA used for this test were also obtained from RITM. The extraction was performed using five (5) mosquitoes with PBS as grinding medium. One hundred (100 µL) DNAzyme-AuNP was mixed with 20 µL of each extracted RNA, 10 µL 10 mM $MgCl_2$ and 10 µL 1.0 M NaCl. The experiment was performed in duplicates. The picture of the results was taken after 20 min of reaction time and is shown in Figure 10.

Qualitative observations showed that only the extracted DENV-3 RNA produced a positive result evidenced by a color change of pink to purple. The mixtures containing the other dengue serotypes as well as the pure DNAzyme-AuNP solution remained pink. The summary of the results are tabulated in Table 4.

Since the sequence of the detector DNAzyme was designed specifically for a specific area in the DENV-3

genome, the detector DNAzymes were expected to bind only to this specific sequence unique to the DENV-3 genome. The results showed that the detector DNAzymes can successfully distinguish DENV-3 from the other serotypes.

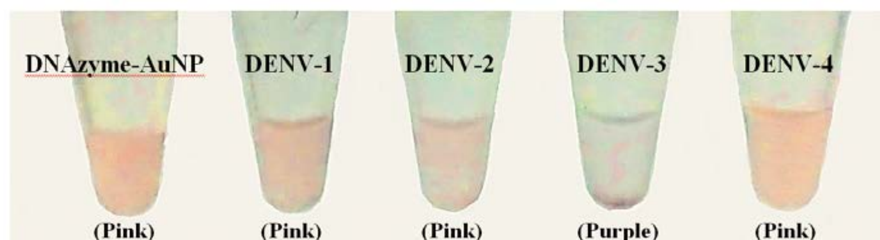


**Figure 10:** Selectivity Analysis using extracted RNA from *Aedes aegypti*.

**Table 4:** Summary of the selectivity of the colorimetric detection of DENV-3.

| Label | Color | Result (+/-) |
|---|---|---|
| DENV-1 | pink | - |
| DENV-2 | pink | - |
| DENV-3 | purple | + |
| DENV-4 | pink | - |

The absorbances of the mixtures at 520 nm were also determined using UV/Vis Spectrophotometry. Based on Figure 16, a significant decrease was observed on the samples containing the extracted DENV-3 RNA in comparison with the pure DNAzyme-AuNP solution. The mixtures containing the other dengue serotypes produced absorbance values that were not significantly different from that of the pure DNAzyme-AuNP solution which supports the qualitative observations that no significant reaction took place. These results together with the qualitative ones demonstrate that the DNAzyme-AuNP based sensor was specific for DENV-3.

Studies have showed that a person that previously suffered from dengue infection caused by one dengue serotype is immune to that particular serotype. Within two to three months after recovering from the dengue infection caused by one serotype, a person is immune from infection caused by the other three serotypes. After the said period, that person is susceptible to dengue infection again. It was also reported by Dr. Scott Halstead that people who were exposed to a second dengue infection have a greater risk of developing severe dengue compared to those who had not been infected before [6].

### *3.6 Effect of Varying Target Concentration on the Absorbance of Aggregated Gold Nanoparticles*

The effect of varying target concentration on the absorbance of aggregated gold nanoparticles was determined using two set-ups. The first set-up involved the use of synthetic DENV-3 target oligonucleotide while the second involved the use of extracted RNA from *Aedes aegypti*. Different concentrations of the synthetic target was prepared and tested on the nanoparticle-based sensor. Six concentrations were used (0.001 to 100 μM) and the same procedure for the colorimetric detection was performed. One hundred (100 μL) DNAzyme-AuNP was

mixed with 20 µL of each concentration, 10 µL 10 mM $MgCl_2$ and 10 µL 1.0 M NaCl and were incubated at room temperature. Results of the analysis were gathered after 20 min of reaction time and are shown in Figure 12.

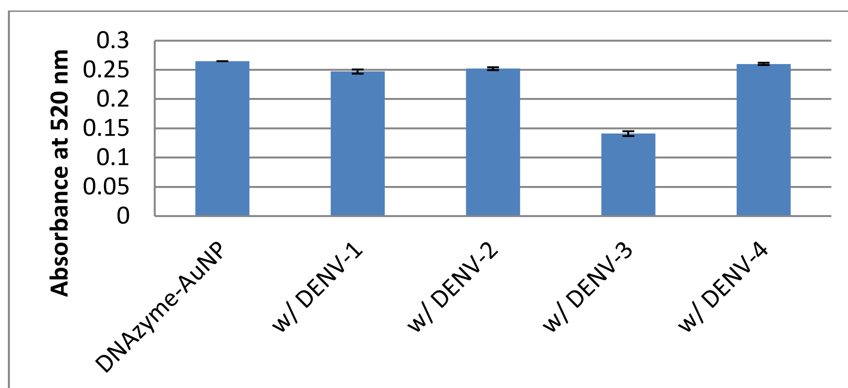


**Figure 11:** Quantitative analysis of the selectivity of the DNAzyme-AuNP based sensor for DENV-3 using UV/Vis spectrophotometry.

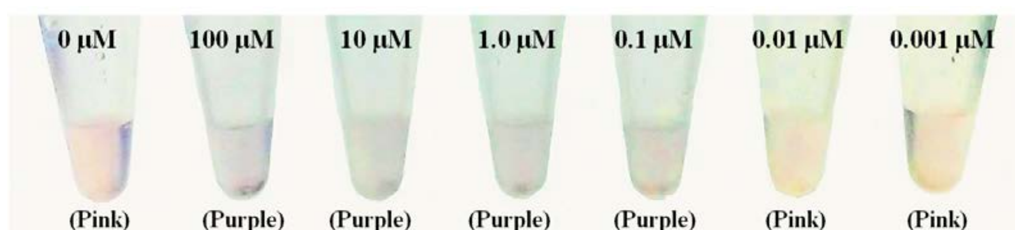


**Figure 12:** Determination of the effect of varying synthetic DENV-3 target concentration on the resulting color of the DNAzyme-AuNP solution.

Based on the resulting color of the solutions, 0.1 µM was the lowest concentration that the sensor can detect since it is the lowest concentration that caused a color change of pink to purple indicating a positive reaction. Lower concentrations (0.01 and 0.001 µM) remained pink which means the sensor can no longer detect those concentrations to give a visible reaction. The summary of the results is given in Table 5.

**Table 5:** Summary of the results on the effect of varying synthetic DENV-3 target concentration on the resulting color of the DNAzyme-AuNP solution.

| Label | Color | Result (+/-) |
|---|---|---|
| 100 µM | purple | + |
| 10 µM | purple | + |
| 1 µM | purple | + |
| 0.1 µM | purple | + |
| 0.01 µM | pink | - |
| 0.001 µM | pink | - |

At very low concentrations, although the DNAzymes were still able to cleave the RNA in the presence of $Mg^{2+}$ ions, the activity was not enough to cause deshielding of the gold nanoparticles hence no aggregation was observed. The concentration may be too low that the cleavage activity has almost negligible effect on the gold nanoparticles that is why the mixture remained pink.

The samples were further analyzed using UV-Vis Spectrophotometry after 30 min of incubation. The absorbances of the samples at 520 nm were graphed with respect to the concentration of DENV-3 used. The graph in Figure 13 showed that the highest concentration of the synthetic target gave the lowest absorbance. At decreasing concentrations of the target DENV-3, the absorbances increased until it reached 0.01 µM and 0.001 µM where the absorbances were almost similar to each other and the pure DNAzyme-AuNP solution without the synthetic DENV-3 target. This is because at these two concentrations, the solutions remained pink hence no aggregation was observed. At 5% by LSD, the lowest target concentration that the nanobiosensor can detect that was significantly different from the blank (0 µM) was 0.1 µM. The lowest concentration that the sensor can detect is still relatively high for other studies have reported to detect up to 0.06 nM of the virus [9].

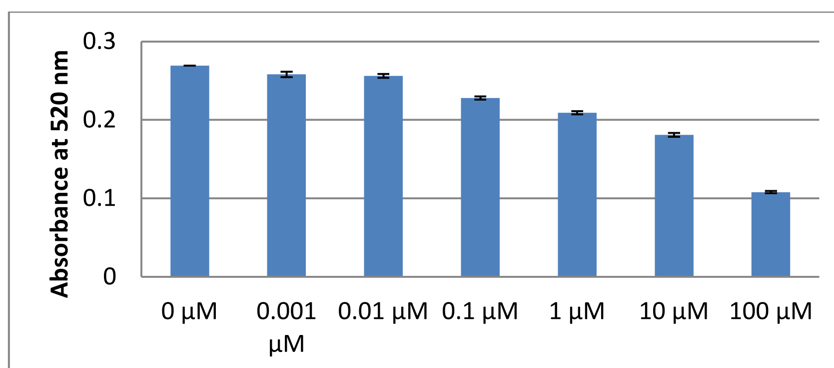


**Figure 13:** Quantitative analysis on the effect of varying synthetic DENV-3 target concentration on the absorbance of aggregated gold nanoparticles.

Determination of the effect of varying target concentration on the absorbance of aggregated gold nanoparticles was also performed using different concentrations of DENV-3 RNA extracted from *Aedes aegypti*. The concentrations used were 5 x $10^{-1}$ to 5 x $10^{4}$ PFU/mL. Twenty (20) µL of each concentration was tested with 100 µL DNAzyme-AuNP, 10 µL 10 mM $MgCl_2$ and 10 µL 1.0 M NaCl and was incubated at room temperature for 20 min. Results of the test are shown in Figure 14.

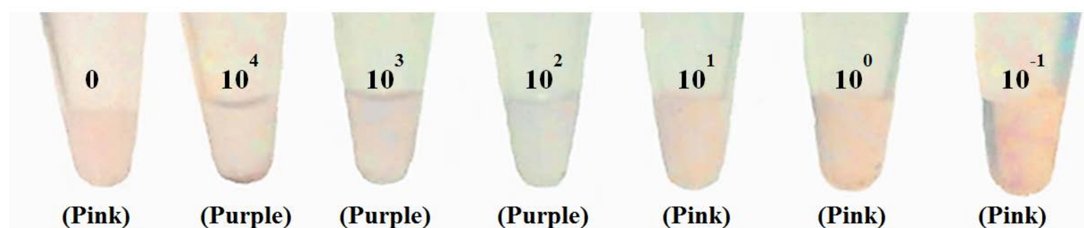


**Figure 14:** Effect of varying extracted target RNA concentration on the resulting color of the DNAzyme-AuNP solution.

Based on qualitative observations, the DNAzyme-AuNP based sensor was able to detect up to 5 x $10^2$ PFU/mL of DENV-3 RNA. The summary of the results of the experiment are tabulated in Table 6.

**Table 6:** Summary of the results on the effect of varying extracted target RNA concentration on the resulting color of the DNAzyme-AuNP solution.

| Label | Color | Result (+/-) |
|---|---|---|
| $10^4$ | purple | + |
| $10^3$ | purple | + |
| $10^2$ | purple | + |
| $10^1$ | pink | - |
| $10^0$ | pink | - |
| $10^{-1}$ | pink | - |

UV/Vis Spectrophotometry was also applied in the analysis resulting to the graph in Figure 15. Based on the observed trend, only concentrations starting from 5 x $10^2$ PFU/mL gave absorbance values that were significantly lower than the blank. Concentrations from 5 x $10^1$ to 5 x $10^{-1}$ PFU/mL showed absorbance values that were not significantly different from the blank (0 PFU/mL).

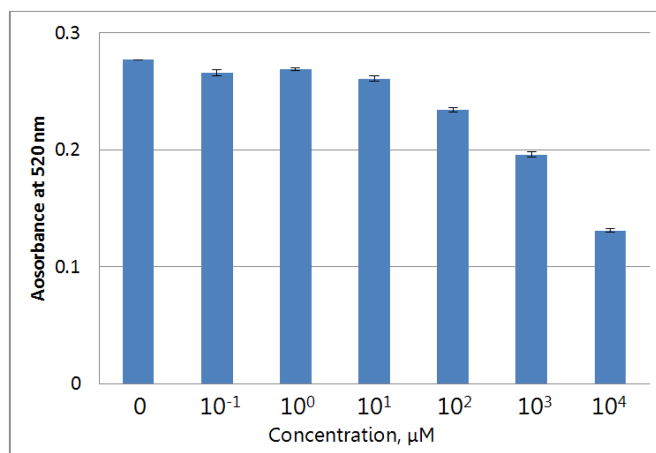


**Figure 15:** Quantitative analysis on the effect of varying extracted target RNA concentration on the absorbance of aggregated gold nanoparticles.

The lowest concentration of the dengue virus that the RT-PCR method used in RITM can detect is 5 x $10^{-2}$ PFU/mL which is greatly lower than the obtained lowest concentration of the nanoparticle-based sensor in this study which is 5 x $10^2$ PFU/mL. This aspect of the sensor can still be improved since the gold nanoparticle solution used in this study was diluted (1:10 dilution). By using a more concentrated solution, more gold nanoparticles will be present in the reaction mixture giving a more intense color of the solution. Also, more DNAzyme molecules will be present that can cleave more DENV-3 RNA molecules hence improvement on the lowest concentration that the nanobiosensor can detect is expected.

The paragraphs continue from here and are only separated by headings, subheadings, images and formulae. The section headings are arranged by numbers, bold and 10 pt. Here follows further instructions for authors.

## 4. Conclusion

A nanoparticle-based sensor was fabricated for the detection of dengue virus serotype-3 (DENV-3) using DNAzyme-functionalized dextrin-capped gold nanoparticles (DNAzyme-AuNP). The colorimetric detection uses the enzymatic activity of DNAzymes. Upon binding with the target sequence found uniquely in the genome of the DENV-3 RNA, the DNAzyme cleaves the RNA molecule in the presence of $Mg^{2+}$ ions. This activity causes deshielding of the nanoparticles from each other. With the aid of NaCl, aggregation of the nanoparticles occur which can be visually observed as a color change from pink to purple. This reaction can be quantitatively determined by measuring the absorbance of the mixtures at 520 nm using UV/Vis Spectrophotometry. Mixtures that gave a positive result by turning purple gave absorbance values significantly lower than the DNAzyme-AuNP solution without any target RNA.

The nanobiosensor was tested against both synthetic DENV-3 target oligonucleotide and extracted RNA from *Aedes aegypti*. For the latter, two extraction methods were applied which used PBS and Trizol as the grinding medium. The extraction method using PBS proved to be more suitable for this type of analysis since the other extraction method which uses Trizol gave a false negative result due to the interference of the color of Trizol.

The DNAzyme-AuNP based sensor was also determined to be specific for DENV-3 by testing it with the other dengue serotypes. The extracted RNA from the other dengue serotypes were used for the analysis. Qualitative observations as well as spectrophotometric analysis showed that the sensor was indeed specific for DENV-3. The effect of varying target concentration on the absorbance of aggregated gold nanoparticles was also determined using synthetic DENV-3 target oligonucleotides and extracted RNA from *Aedes aegypti*. Using the synthetic DENV-3 target, the sensor was determined to be able to detect up to 0.1 μM of the target RNA. The results were further analyzed using UV/Vis spectrophotometry and showed that increasing concentrations of DENV-3 on the samples that produced a positive result gave decreasing values of absorbance. Using extracted RNA from *Aedes aegypti*, the fabricated sensor was determined to be able to detect up to $5 \times 10^{2}$ PFU/mL, which in comparison with that of RITM which is $5 \times 10^{-2}$ PFU/mL, was still high. The lowest concentrations that the sensor can detect using both synthetic DENV-3 target and extracted RNA may not be as low as the currently used methods but this feature of the sensor can still be improved through modifications in the procedure. It is recommended to use a more concentrated solution of the synthesized dextrin-capped gold nanoparticles in future experiments to provide a more intense color of the solution for the qualitative observations. Improvement on the lowest concentration that the nanobiosensor can detect is also expected with this modification due to the presence of higher amounts of the gold nanoparticles conjugated to DNAzymes that can detect DENV-3 in the reaction mixture. Also, other parameters such as storage life and temperature resistance of the functionalized nanoparticles can be studied to provide better assessment of its stability.

The results of the study demonstrated that the dextrin-capped gold nanoparticles were successfully utilized for the colorimetric detection of DENV-3 in *Aedes aegypti*. The use of synthesized gold nanoparticles instead of

purchased ones also provides a cheaper and "greener" alternative for the fabrication of the dengue sensor. This study shows promising applications of the synthesized gold nanoparticles for dengue detection since it provides a simple, portable, "greener" and faster way of detecting DENV-3 in mosquitoes.

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**References**

[1] Centers For Disease Control And Prevention. 2014. Dengue. Retrieved July 22 2014 from http://www.cdc.gov/dengue

[2] M.G. Guzman, S.B. Halstead, H. Artsob, P. Buchy, M. Farrar, D.J. Gubler, E. Hunsperger, A. Kroeger, H.S. Margolis, E. Martínez, M.B. Nathan, J.L. Pelegrino, C. Simmons, S. Yoksan, R.W. Peeling, "Evaluating Diagnostics Dengue: a continuing global threat". 2010. Macmillan Publishers Limited. doi:10.1038/nrmicro2460

[3] World Health Organization (WHO). 2014. "Dengue and severe dengue." Fact sheet Nº117. Retrieved July 2 2014 from http://www.who.int/mediacentre/factsheets/fs117/en/

[4] Department Of Health: National Epidemiology Center. "Public Health Surveillance And Informatics Division." 2013. Disease Surveillance Report. Morbidity Week 36, September 1-7, 2013. Philippines. Department of Health.

[5] L. Bravo, V.G. Roque, J. Brett, R. Dizon, and M. L'azou. "Dengue virus serotype distribution in the Philippines: regional studies", PLOS Neglected Tropical Diseases.10.1371/journal.pntd.0003027.g004.

[6] S.B. Halstead. (Ed). 2008. "Dengue." Tropical Medicine: Science and Practice Vol 5. Singapore. Imperial College Press. p. 75-110

[7] A. Jittmittraphap, S. Thammapalo, N. Ratanasetyuth, N. Wongba, M.P. Mammen, and W. Jampangern. "Rapid Detection of Dengue Viral RNA in Mosquitoes by Nucleic Acid-Sequence Based Amplification (NASBA)". 2006. Southeast Asian J Trop Med Public Health. Vol 37 No. 6 November 2006

[8] P. Samuel and B.K. Tyagi. "Diagnostic methods for detection & isolation of dengue viruses from vector mosquitoes". 2006. Indian J Med Res 123, May 2006, pp 615-628

[9] J.R. Carter, V. Balaraman, C.A. Kucharski, T.S. Fraser and M.J. Fraser Jr. "A novel dengue virus

detection method that couples DNAzyme and gold nanoparticle approaches". 2013. Virology Journal 2013, 10: 201.

[10] R.W. Peeling, H. Artsob, J.L. Pelegrino, P. Buchy, M.J. Cardosa, S. Devi, et.al. "Evaluation of diagnostic tests: Dengue". 2010. World Health Organization. Macmillan Publishers Limited. doi:10.1038/nrmicro2459

[11] S. Tang and I. Hewlett. "Nanoparticle-Based Immunoassays for Sensitive and Early Detection of HIV-1 Capsid (p24) Antigen". 2010. The Journal of Infectious Diseases 2010; 201(S1):S59–S64 DOI: 10.1086/650386

[12] P.C. Ray, S.A. Khan, Z. Fan, and D. Senapati. "Gold Nanotechnology for Targeted Detection and Killing of Multiple Drug Resistant Bacteria from Food Samples". 2013. Advances in Applied Nanotechnology for Agriculture Chapter 1, pp 1–19 doi: 10.1021/bk-2013-1143.ch001

[13] F. Li, Q. Zhao, C. Wang, X. Lu, X.F. Li, and X. Le. "Detection of Escherichia coli O157:H7 using gold nanoparticle labeling and inductively coupled plasma mass spectrometry". 2010. Anal Chem. 2010 Apr 15;82(8):3399-403. doi: 10.1021/ac100325f.

[14] M. Anderson, E. Torres-Chavolla, B. Castro,and E. Alocilja. "One step alkaline synthesis of biocompatible gold nanoparticles using dextrin as capping agent" J Nanopart Res DOI 10.1007/s11051-010-0172-3

[15] J. Liu and Y. Lu. "Preparation of aptamer-linked gold nanoparticle purple aggregates for colorimetric sensing of analytes", 2006. Nat Protoc 2006, 1:246–252.

[16] D. Zhang, M.C. Huarng and E.C. Alocilja. "A multiplex nanoparticle-based bio-barcoded DNA sensor for the simultaneous detection of multiple pathogens". 2010. Biosens Bioelectron. 2010 Dec 15;26(4):1736-42. doi: 10.1016/j.bios.2010.08.012. Epub 2010 Aug 11.

[17] R.L. Whetten and R.C. Price. Chemistry: Nano-Golden Order. 2007. Science. 318:407– 408. doi: 10.1126/science.1150176

[18] A. Ogawa and M. Maeda. "Easy design of logic gates based on aptazymes and noncrosslinking gold nanoparticle aggregation". 2009. Chem Commun (Camb) 2009, 21:4666–4668.