

A DNA-Based Nanobiosensor for the Rapid Detection of the Dengue Virus in Mosquito

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Abstract—This paper describes the development of a DNA-based nanobiosensor to detect the dengue virus in mosquito using electrically active magnetic (EAM) nanoparticles as concentrator and electrochemical transducer. The biosensor detection encompasses two sets of oligonucleotide probes that are specific to the dengue virus: the detector probe labeled with the EAM nanoparticles and the biotinylated capture probe. The DNA targets are double hybridized to the detector and the capture probes and concentrated from nonspecific DNA fragments by applying a magnetic field. Subsequently, the DNA sandwiched targets (EAM-detector probe–DNA target–capture probe–biotin) are captured on streptavidin modified screen printed carbon electrodes through the biotinylated capture probes. Detection is achieved electrochemically by measuring the oxidation–reduction signal of the EAM nanoparticles. Results indicate that the biosensor is able to detect the redox signal of the EAM nanoparticles at dengue DNA concentrations as low as 10 ng/ μ l.

Keywords—Dengue, magnetic nanoparticles, mosquito, nanobiosensor.

I. INTRODUCTION

DENGUE is a deadly disease transmitted mostly from mosquito bites. Viral outbreaks have spread vastly across the world mostly in warm climate areas. This mosquito-borne viral disease spread in developing countries due to substandard housing, inadequate waste and water management, immigration, airborne travel, and deteriorating disease prevention programs [1]. Each year, there are approximately 100 million cases of dengue fever or dengue hemorrhagic fever worldwide. Dengue is currently one of the most important arthropod-borne diseases and may be caused by four different dengue virus serotypes (DENV-1 to DENV-4), transmitted mainly by *Aedes aegypti* (Diptera: *Culicidae*) mosquitoes. With the lack of a dengue vaccine, vector control strategies constitute a crucial mode to prevent or reduce disease transmission. In this context DENV detection in natura *Ae. aegypti* populations may serve as a potential additional tool for early prediction systems of dengue outbreaks, leading to an intensification of vector control measures, aimed at reducing disease transmission.

Disease prevention and control measures have been established for early detection and monitoring of dengue

outbreaks. Innovative solutions have been developed to combat outbreaks. Unfortunately, the lack of organized resources and capital in some countries has resulted in a number of increasing dengue viral outbreak cases [2]. Cost effective measures to accurately identify dengue can be combined with rigorous efforts to adequately treat patients and reduce the number of mosquito breeding sites. Accurate diagnosis of infection and effective preventive measures can reduce the number of outbreaks by as much as 30% [OPS/HCP/HCT/].

In developing countries such as the Philippines, cases of dengue virus infection are increasing while dengue detection kits are not affordable or always accessible. Thus traditional approaches are still used that are slow to diagnose and treat in time. In 2013, eight (8) provinces were found of patients having both mosquito-borne diseases chikungunya and dengue. These provinces are now the focus of a special investigation by the Department of Health (DOH). Doctors might not easily distinguish the rarely fatal chikungunya from the often deadly dengue in the first five days of the illness. During the first half of the year, there was a total of 2,594 suspected chikungunya nationwide but only 157 were confirmed as such, according to DOH data.

Laboratory tests for the detection of mosquitoes infected with dengue viruses include isolation of the virus and demonstration of a specific viral antigen or ribonucleic acid (RNA). Isolation of the virus is the most definitive approach, but the techniques involved require a relatively high level of technical skill, equipment, and are time-consuming [3], whereas direct detection of dengue antigen, such as dengue antigen-capture enzyme-linked immunoglobulin assay (ELISA), is not sensitive and has high rates of false positives [4]. Thus, detection of nucleic acid is an alternative method to detect infected mosquitoes. Polymerase chain reaction (PCR) is one technique available for the laboratory diagnosis of dengue infection. This molecular technique is rapid, highly sensitive and specific. However, PCR must still be viewed as an experimental approach because a consensus has not been reached on the optimal standardization of each step of the procedure, and the requirement for a thermocycler which is relatively expensive equipment.

A method based on nucleic acid sequence-based amplification (NASBA) has been developed to detect dengue viral RNA. NASBA is an isothermal RNA amplification technique that is achieved by the reaction of 3 enzymes; usually avian myeloblastosis-reverse transcriptase (AMV-RT), T7-RNA polymerase, and RNase-H [5]. The amplification products can be detected by agarose gel-electrophoresis or

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electrochemiluminescence (ECL). This method gives 100% sensitivity, 96.30% specificity, and 98.15% efficacy for serum samples. NASBA is, therefore, useful in detecting dengue virus infection. Mosquito cell cultures offer a good degree of specificity for dengue virus detection. Mosquito inoculation techniques have been reported for detection and propagation of flaviviruses [6]. Our research proposal aims to develop a nucleic acid-based nanobiosensor for the rapid and specific detection, to differentiate from chikungunya, of the dengue virus in mosquito for epidemiological application.

II. PROCEDURE

A. Chemicals and Reagents

Aniline, iron (III) oxide nanopowder, sodium phosphate (monobasic and dibasic), sodium acetate, phosphate buffered saline tablets (0.01 M), formamide, sodium dodecyl sulfate (SDS), hydrochloric acid (HCl), EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride), Trizma base and streptavidin from *Streptomyces avidinii* were purchased from Sigma Aldrich (St. Louis, MO).

B. DNA Probe Labeling with EAM Nanoparticles

The EAM NPs (kindly provided by Dr. Evangelyn C. Alocilja, Michigan State University) were labeled with the phosphorylated detector DNA probes (Ph-PRO) using phosphoramidate linkage between the amine groups of the polymer and the phosphate group of the oligonucleotides [7]. The NPs were dispersed in sodium acetate buffer by sonication and mixed with the Ph-PRO probes and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC). The mixture was incubated with shaking in a rotational hybridization oven at room temperature. The Ph-PRO DNA probe labeled EAM NPs (PRO-EAMs) were then separated by a magnetic separator and washed repeatedly with acetate buffer and DNase RNase free water to remove the unbound probes.

The conjugation efficiency of the DNA probes with the NPs was determined using 6-carboxy fluorescein (6-FAMTM) labeled Ph-PRO probes (at 3'-end). Attachment of Ph-PRO detector probes to the EAM NPs was confirmed by fluorescence measurements in a Microplate Fluorometer Reader (Victor3, Perkin Elmer, MA). Fluorescence of the pure 6-FAMTM labeled Ph-PRO probe solution and the supernatant solution containing the unreacted probes after magnetic separation of the PRO-EAMs was observed by excitation at 495nm and detection of emission at 520 nm [8].

C. Dual Hybridization and Concentration of Target DNA

The synthetic dengue 2 (DENV 2) oligonucleotide targets were designed based on BLAST search of the DNA sequence of dengue virus 2 from *Aedes aegypti* and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The DENV 2 serotype was selected due to its prevalence in Southeast Asia including the Philippines. The synthetic target DENV 2 oligonucleotide was hybridized dually with the detector probe labeled EAM NPs (PRO-EAMs) and the biotinylated capture probes (PRO-Bio). The DENV 2 target

and appropriate concentrations of the PRO-EAMs and the PRO-Bio probes were hybridized at 45°C for 1 h in a rotational hybridization oven. The resulting EAM–target–biotin DNA hybrids were washed twice with tris-ethylene diamine tetraacetic acid (TE) buffer by magnetic separation of the EAM NPs to remove the unbound target DNA. This was followed by washing the EAM NPs with formamide in phosphate buffered saline (PBS) by magnetic separation to remove the nonspecifically bound DNA targets. Finally, the concentrated EAM–target–biotin DNA hybrids were resuspended in 40 ul of DNase RNase free water.

D. Sensor Design and Surface Modification

A screen printed three-electrode sensor (Gwent Group, UK) was used in the electrochemical detection of DNA targets. The working electrode surface was modified with streptavidin for target binding. The electrodes were then dried at ambient temperature. Electrochemical detection and characterization of straight EAM NPs were performed on the bare working electrode surface using cyclic voltammetry (CV) in a Potentiostat/Galvanostat (Princeton Applied Research, OakRidge, TN) with two vertex potentials in the ramp mode. The concentrated DNA hybrids were allowed to react with the avidin modified electrode surface for 15 min at room temperature and rinsed with DNase RNase free water three times to remove any unbound EAM NPs from the surface and dried at ambient temperature for 15 min. One hundred microliters of 0.1M HCl solution were added to the electrode surface and allowed to equilibrate for 5min. The CV scans were performed using the same scanning potential window as that of the bare EAMs at a scan rate of 20 mV/s [8].

E. Biosensor Sensitivity Analysis

For sensitivity analysis, several concentrations of the synthetic DENV 2 oligonucleotide target were prepared using DNase RNase free water. Each concentration was then dually hybridized with the PRO-EAM and the PRO-Bio probes and the DNA hybrids were electrochemically detected on the streptavidin modified screen printed electrode surface. A blank control consisting of straight EAM NPs (concentration: 10 mg/ml) suspended in DNase RNase free water was tested for comparison. Base line curves for the bare working electrode and the streptavidin.

III. RESULTS AND DISCUSSION

A. Biosensor Detection Principle

The principle of detection of the EAM based electrochemical DNA biosensor is described in [8]. The detection involves an electrochemical sandwich assay engaging a detector DNA probe and a capture DNA probe. The detector probe is labeled with EAM NPs (PRO-EAM) and the capture probe is labeled with biotin (PRO-Bio). The DNA targets are dually hybridized with the PRO-EAM and the PRO-Bio probes resulting in EAM–target–biotin DNA hybrids. The DNA hybrids are concentrated and separated from other noncomplementary sequences and unreacted DNA using a magnetic separation stand. The concentrated DNA

target hybrids are then added directly to the surface of streptavidin modified screen printed electrodes for anchoring the hybrids on the electrode surface using streptavidin–biotin interactions. After a brief incubation period, the electrode surface is washed to remove the excess EAM NPs and the unbound DNA hybrids. The target DNA is finally detected electrochemically on the electrode surface exploiting the redox properties of the EAM NPs.

B. Probe Labeling of the EAM Nanoparticles

The biomodification of the EAM NPs with DNA probes (Ph-PRO) was confirmed by fluorescence and spectrophotometric studies. Table I shows the fluorescence intensity measurements of the pure 6-FAM labeled Ph-PRO probe solution (94, 715) and that of the unreacted probes in the supernatant after magnetic separation (24,590) of the PRO-EAMs (probe labeled EAMs) at different concentrations of the NPs. The supernatant from the labeling process (24,590) after the magnetic separation of the PROEAMs shows significantly lower fluorescence signal than that of the pure probe (94, 715) thus indicating the attachment of the probes to the NPs. This data confirms the attachment of the probes to the EAM NPs.

TABLE I
FLUORESCENCE MEASUREMENTS FOR CONJUGATION EFFICIENCY OF PANI WITH OLIGO PROBES

Sample	Fluorescence (0.1 s) intensity
Acetate buffer	94.50
EAMNP in acetate buffer	83.00
EAMNP mixed with the pure Phos-probe in acetate buffer	94,715.00
EAMNP functionalized with the Phos-probe in acetate buffer	24,590.00

C. Detection of the Dengue 2 Virus

Fig. 1 shows the electrochemical response of the concentrated EAM–target–biotin DNA hybrids using two (2) concentrations of the EAMNP captured on streptavidin modified working electrode following dual hybridization. As evident, the CV of the 10 mg/mL EAMNP is higher than that of the 1 mg/mL EAMNPs. The anodic peak potentials are located at +0.110 and +0.597 V, whereas the cathodic peak potentials are located at +0.530 and –0.070 V. The presence of the redox peaks in the CV response demonstrates that the EAMNPs are electrochemically active after the probe labelling and sandwiched hybridization processes and also confirms the dual function of the EAM NPs, i.e. successful magnetic capture of target DNA and electrochemical detection of the concentrated EAM–target–biotin DNA hybrids.

D. Sensitivity Analysis of DENV 2 Target

Fig. 2 shows the electrochemical response of the biosensor in DENV 2 target concentrations ranging from 10 to 1000 ng/ μ L and the blank (control). The blank solution consisted of probe labeled EAM NPs hybridized with 0 ng/ μ L of the DENV 2 target solution.

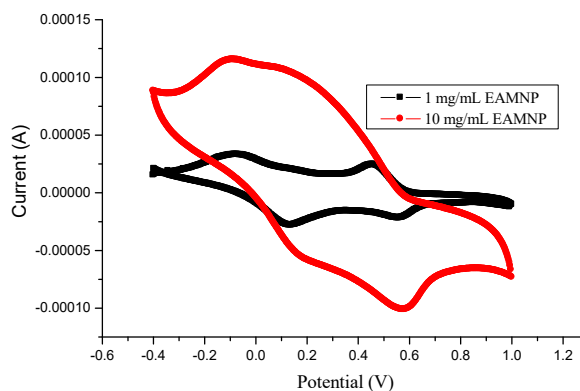


Fig. 1 Effect of EAMNP concentration on the electrochemical response of the DENV nanobiosensor

As observed in the CV response, the two characteristic redox peaks of the EAM NPs are present at different concentrations of the DENV 2 target. It is apparent that an inverse relationship between target concentration and current is observed using this method: as the target concentration decreases, the current response increases. This observation may be the result of interparticle distances. At low concentrations, the particles are more dispersed and have more accessible surface area to interact with the acidic solution producing slightly higher peak current the presence of EAM redox peaks in the CV scans also confirms the ability of the EAM NPs to capture and detect oligonucleotide targets from low DNA concentrations.

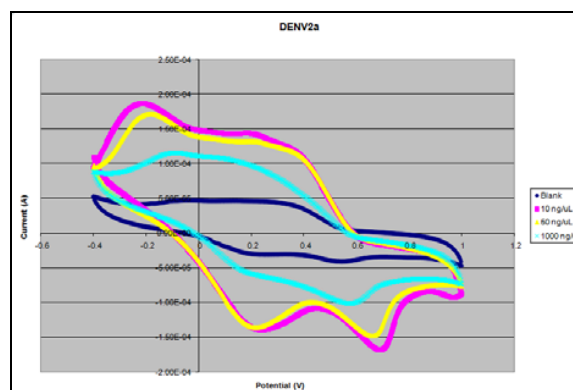


Fig. 2 Electrochemical response of various concentrations of the DENV 2 target during detection using the fabricated dengue nanobiosensor

Aedes aegypti (Linnaeus) is a widely distributed mosquito and the main urban vector involved in dengue virus transmission throughout the world, including the Philippines. Demographic and social changes including unplanned urbanization, increasing population size, as well as ineffective mosquito control measures in most dengue endemic regions of the world have contributed to broaden the geographical distribution of this mosquito species [9], [10]. The long-term plan for this study is as tool for the early prediction of the

dengue virus in natural *Aedes* populations prior to dengue outbreak particularly in developing countries. The research team has continuing experiments on mosquito samples collected from stagnant waters in rural communities in the Philippines (Fig. 3).



Fig. 3 On-site sampling and analysis of mosquito larvae using fabricated nanobiosensor for dengue virus detection. *Inset*. Prototype of the fabricated dengue virus nanobiosensor with the handheld potentiostat

IV. CONCLUSION

This study explores the potential of EAM nanoparticles as a magnetic concentrator of DNA targets and a novel nanostructured transducer in the electrochemical detection of DNA. The EAM based DNA biosensor is fabricated and implemented in the detection of the dengue virus 2 in mosquito. The sensitivity of the biosensor as determined from cyclic voltammetry measurements is 10 ng/ μ l of the DNA target in a total detection time of 60 min. These results indicate that the EAM nanoparticles hold promise in electrochemical biosensing applications. Future research work would involve evaluating the specificity of the biosensor and advancing the detection process through different electrochemical techniques. Furthermore, the potential of the biosensor as a field based device for diagnostic and biosecurity applications will also be explored.

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REFERENCES

- [1] M. Guszman and G. Kouri. Dengue and dengue hemorrhagic fever in the Americas: lessons and challenges. *Journal of Clinical Virology*. 2003. 27: 1-13.
- [2] F. Pinheiro. Dengue in the Americas. 1980-1987. *Epidemiol Bull PAHO*. 1989; 10:1-8.
- [3] D.J. Gubler. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol*. 2002. 10: 100-3.
- [4] R. Sithiprasasna, D. Strickman, B.L. Innis, K.J. Linthicum. ELISA for detecting dengue and Japanese encephalitis viral antigen in mosquitoes. *Ann Trop Med Parasit*. 1994. 88: 397-404.
- [5] J. Compton. Nucleic acid sequence-based amplification. *Nature*. 1991. 350: 91-2.
- [6] P.P. Samuel, B.K. Tyagi. Diagnostic methods for detection & isolation of dengue viruses from vector mosquitoes. *Indian J. Med. R*. 2006. 123(5): 615-28.
- [7] Zhu, N.N., Chang, Z., He, P.G., Fang, Y.Z. 2006. *Electrochimica Acta*. 51 (18), 3758–3762.
- [8] Pal, S. and Alocilja, E.C., 2010. Electrically-active magnetic nanoparticles as novel concentrator and electrochemical redox transducer in *Bacillus anthracis* DNA detection. *Biosensors and Bioelectronics*. 26(4), 1624-1630.
- [9] Chow VT, Chan YC, Yong R, Lee KM, Lim LK, Chung YK, et al. Monitoring of dengue viruses in field-caught *Aedes aegypti* and *Aedes albopictus* mosquitoes by a type-specific polymerase chain reaction and cycle sequencing. *Am J Trop Med Hyg* 1998; 58: 578–86.
- [10] Fouque F, Garinci R, Gaborit P. Epidemiological and entomological surveillance of the co-circulation of DENV-1, DENV-2 and DENV-4 viruses in French Guiana. *Trop Med Int Health* 2004; 9: 41–6.