Title: Colorimetric Sensor Devices Using Aptamer-Gold Nanoparticle Conjugates for Field Surveillance of Mosquito-Borne Diseases

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ABSTRACT

There is an unmet need for passive (unpowered) mosquito field surveillance tools that can provide straightforward readouts in real-time to indicate the presence of vector mosquito populations and mosquito-borne pathogens. We are therefore spearheading the development of new diagnostic tools and sensors with colorimetric readouts based on DNA aptamer-gold nanoparticle conjugates (Apt-AuNPs). The focus in this pioneering study was to identify *Aedes (Ae.) aegypti* mosquitoes via specific detection of *Ae. aegypti* salivary proteins in a sugar feeding solution. Salivary proteins were first collected either directly or via salivary gland extracts (soluble fraction only), purified and then used as complex targets for the synthetic generation of DNA aptamer molecules. Fifteen (15) new, unique aptamers that specifically bound *Ae. aegypti* salivary proteins were created using this approach. These aptamers were then coupled to gold nanoparticles and screened against a major salivary protein, D7 (recombinant), and the soluble salivary gland extracts. Four (4) salivary protein binding aptamers were selected for further experiments based on the UV/Vis spectroscopic results of the first screen. Four (4) different Apt-AuNPs prepared with selected aptamers were able to detect D7 protein levels in sugar solutions as low as ~10ng via UV/Vis spectroscopy and produced visible color changes (red to purple) in sugar solutions containing as little as ~40ng of D7. These are the first Apt-AuNPs designed to and specifically capable of colorimetrically detecting a major protein component of *Ae. aegypti* saliva. These novel diagnostics developed in this project are the first key step required for the creation of passive, colorimetric field surveillance sensors for *Ae. aegypti*. More research is required to increase the sensitivity of the Apt-AuNPs to whole mosquito saliva and incorporate them into a paper/membrane-type sensor.
INTRODUCTION

Florida mosquito control districts are one of the primary lines of defense against mosquito-borne illnesses that threaten public health and safety. These districts utilize a range of tools and methods to carry out their responsibilities such as public education, mosquito surveillance traps, applications of larvi- and insecticides, molecular biological testing of collected specimens and sentinel chicken programs. However resources vary between districts and all have relatively limited resources in relation to the geographic areas for which they are responsible and the unique threats and challenges faced/posed therein. District managements must therefore routinely make decisions on how to best deploy limited resources to most effectively protect public health. Like all decisions, accurate, up-to-date information is key to making good decisions. For mosquito control districts, knowledge about the presence/distribution of vector mosquitoes such as Aedes (Ae.) aegypti, and pathogens like dengue, chikungunya and Zika viruses (DENV, CHIKV and ZIKV respectively) in a given area is crucial to this decision making. Therefore, a set of field surveillance tools capable of detecting the presence of vector mosquito populations and associated pathogen in real-time via a straightforward readout such as color change would be powerful additions to the toolboxes of mosquito control districts.

This project was the initial step in developing these powerful new mosquito field surveillance tools and the first goal was to develop a new set of colorimetric diagnostics to detect Ae. aegypti. Our overall approach—inspired by the seminal work of Hall-Mendelin et al.—was to interrogate salivary proteins that are expectorated during sugar feeding with DNA aptamer-gold nanoparticle conjugates (Apt-AuNPs) developed to bind/detect major salivary proteins, D7 specifically. D7 proteins are a family of odorant-binding proteins that make up to an estimated 20% of expectorated salivary proteins. Aptamers are commonly short sequences of single-stranded nucleic acids, often DNA, that can specifically bind target molecules such as proteins, other nucleic acids and drugs with antibody-like specificity and affinity. Unlike antibodies, aptamers are created via multiple rounds of a synthetic in vitro selection process also called systematic evolution of ligands by exponential enrichment (SELEX) whereby a large initial random library of oligonucleotide sequences is checked for binding to a target; those sequences that bind are isolated (i.e., selected), amplified by PCR and serve as the pool of oligonucleotides for the next round of selection. This process is repeated until the sequence(s) with the highest
specificity and affinity for the target is identified. Large, purified quantities of this newly identified aptamer can then be synthesized entirely in the laboratory using. Compared to antibodies, aptamers to specific targets can be generated more rapidly (several weeks, opposed to months), are stable in a wider range of solutions and temperatures, and can be generated via a completely synthetic process.

Aptamers can also be easily conjugated to gold nanoparticles thereby enabling a colorimetric detection readout for target molecules. Ultimately, our intent is to create a paper/membrane sensor soaked with a sugar solution containing D7-specific Apt-AuNPs. When Ae. aegypti mosquitoes sugar feed from the sensor, D7 proteins in expectorated saliva bind to the Apt-AuNPs and induce a visible color change on the sensor substrate. This color change when the target is absent or present respectively, is due to the relative aggregation of the gold nanoparticles in solution. When target molecules are not present in solution, Apt-AuNPs are dispersed and the solution has a reddish color. When target molecules are present, binding of Apt-AuNPs causes a conformational change in the coupled aptamers that results an increase in the aggregation of the gold nanoparticles thereby changing the solution color to bluish, purple color. This Au-aptamer approach and closely similar has been used recently by others to develop a colorimetric diagnostic assay for malaria with aptamers against the lactate dehydrogenase enzymes of the Plasmodium parasite.

**Methodology**

*Mosquito Rearing (University of Central Florida College of Medicine Mosquito Colony):*

Ae. aegypti eggs obtained from the United States Department of Agriculture-Agricultural Research Service, Center for Medical, Agricultural and Veterinary Entomology (USDA-ARS-CMAVE, Gainesville, FL) were kept on moist crepe paper in air-tight containers in the dark. For each batch, 1,000 eggs (by weight; ~10mg) were brushed off the cards and transferred to 70µm cells strainers, cleaned with 2mL of 0.67M sodium hypochlorite and rinsed with DI water 3×10ml. The eggs were then dispersed into 3L of spring water (Zepherhills, FL) and 25mL of 50g/L 60:40 brown food; liver powder (MP Biologics, Santa Ana, CA) and brewer’s yeast (Insectsales.com) added. Larval trays were incubated at 29°C and brown food added at d3 (12.5mL) and d5 (25mL). At d6 or over 50% pupae, the larvae/pupae were poured and rinsed.
through a 500µm strainer, weighed and up to 4g added to an 8oz. (50cm² surface area) cup with 200mL of spring water and 3mL brown food. The majority of the mosquitoes emerged within 24h after the pour off and d1 was counted then. Sugar water (10% sucrose in DI water) was provided with saturated cotton balls atop of the cages (8×8” Bioquip, Rancho Dominguez, CA) and replaced and rinsed off every 2-3 days.

**Capillary-Tube Direct Saliva Collection:**
Direct saliva collections were performed by the Willenberg team at the USDA-ARS-CMAVE facilities and in the Machain-Williams lab at the Universidad Autonoma de Yucatan Centro de Investigaciones Regionales - Dr. Hideyo Noguchi in Merida, Mexico following protocols similar to those previously reported\(^\text{11}\). Briefly, the mosquitoes were first cold anesthetized and all their wings and legs were removed; they were then stuck down in rows on double-sided tape. Once immobilized, each individual proboscis was placed into glass capillary with a small volume of immersion or mineral oil and the mosquito allowed to salivate into the oil for at least 1h.

**Extraction of Salivary Glands:**
The mosquitoes were first cold anesthetized and all their wings and legs were removed; a small drop of buffer, \(\sim10\mu\text{L}\) of phosphate-buffered saline (PBS), was then applied at the head. Gentle pressure was applied to the upper thorax with the forceps blunt side and with a fine pair of forceps (Miltex, York, PA) behind the head, the neck was grasped and pulled slightly. Keeping the pressure on the thorax, the forceps were re-gripped within the body cavity behind the head, at an angle towards the legs and the head was pulled free. One set of salivary glands was typically attached to the head if not both; the other were either free floating or attached to the connection between the crop and diverticulum which may have come out with the head or was attainable by pulling out tissue from the thorax. The glands and surrounding fluid were collected with 28G1/2” syringes and stored at \(-80^\circ\text{C}\) until enough were collected for protein isolation.

**Protein Isolation, Purification and Characterization:**
For directly collected saliva, the oil layer was vortexed in amicrocentrifuge tube with PBS to extract the proteins from the oil into the PBS. The tube was then centrifuged at 14k rpm for 5 minutes. The water layer was then removed and the extraction and separation processes repeated
one additional time. The PBS layers containing the salivary proteins were pooled and then subjected to the trichloroacetic acid (TCA) protein precipitation/purification procedure described below.

For the isolation and purification of soluble proteins from salivary glands, salivary glands from approximately 200 mosquitoes were pooled and added to gentleMACS™ C Tubes (Miltenyi Biotec Inc, San Diego, CA) and kept on ice. Template B (30 seconds with increasing speed intervals with reverse turns between intervals) was then ran and the tube removed, inverted and mixed gently before a second run. Then the C Tube was centrifuged at 120×g for 5min at 10°C, the contents then filtered through a 70µm cell strainer and centrifuged again at 120×g for 5min at 10°C. The contents were then transferred to a 1.5mL microcentrifuge tube and centrifuged at 10k×g for 5min at 4°C to pellet remaining debris.

The supernatant (or protein-rich PBS from the direct collections) was transferred to a new 1.5ml tube and TCA was added to yield a final concentration of 15-20%. The sample was vortexed for 15sec 3× and incubated at 4°C for 1h, vortexing midway through. Then, the sample was centrifuged at 13.5k×g for 10min at 4°C to pellet the protein. Ice-cold acetone was then added (500µL) and the pellet dislodged. The sample was stored in acetone for at least 1h at -20°C, then centrifuged at 13.5k×g for 10min at 4°C and 2×. The pellet was air dried for 3-5min and resuspended in 1mM PBS at 37°C for 20-30min with pipetting every 5min and then incubated at 4°C overnight. The sample was then centrifuged at 10k×g for 1min at RT to remove the insoluble fraction. 10µL of 1mM PBS is added and pipetted to wash the pellet, centrifuged 10k×g for 1min at RT and the supernatant was added to the first fraction. Protein concentrations are determined by Bradford assay (Thermo Fisher) and ran on a 4-12% Bis-Tris acrylamide gel (Thermo Fisher) (50-500ng). The gel was then washed in water and stained with Pierce™ Silver Stain kit (Thermo Fisher). The gel was dried with DryEase® System (Thermo Fisher).

Sugar Feeding Solution (SFS, i.e., Aptamer Working Solution):
A number of preliminary feeding experiments were conducted to screen for an optimal solution that would facilitate both mosquito feeding and aptamer stability and function. Ultimately it was determined that a solution containing 10% sucrose, 15mM NaCl, 1mM Na₂CO₃, 1µM MgCl₂ (to
aid in aptamer stability) and 5µM β,γ-methylene-ATP (non-hydrolyzable ATP to increase probing and feeding) all at an unadjusted solution pH of ~8.3 met all requirements.

**Salivary Aptamer Synthesis:**
Salivary proteins, both direct collections from Mexico and soluble salivary gland extracts (7.36µg and 8.3µg respectively) were sent to Base Pair Biotechnologies, Inc. (BPB, Pearland, TX) for targets to generate salivary-binding aptamers. SFS was also sent to BPB for the creation of the aptamers with optimal function in the intended end-use chemical environment. At BPB, the targets were input into multiple rounds of their proprietary multiplex SELEX process followed by microarray analysis against recombinant Ae. aegypti D7 protein (MyBioSource, San Diego, CA) to better identify D7-binding aptamers. Fifteen (15) potential candidate aptamers were identified, thiolated (3′) and provided (250nmol each) to the Seal nanoscience team.

**Preparation and Characterization of Gold Nanoparticles (AuNPs):**
Gold nanoparticles (~13nm) were prepared as described elsewhere\textsuperscript{12}. Briefly, aqueous solutions of HAuCl\textsubscript{4} (100ml, 1mM) were reduced using sodium citrate (2ml, 194mM). After mixing the reagents, resultant solutions were refluxed for 20min and allowed to cool at room temperature. Solutions were then passed through a 0.45µm syringe filter to obtain nanoparticles with homogenous size. Dynamic light scattering (DLS) and transmission electron microscopy (TEM) was used to size the AuNPs; surface charge of the nanoparticles was also analyzed using DLS\textsuperscript{13,14}.

**Preparation and Characterization of Salivary Aptamer Gold Nanoparticle Conjugates (Apt-AuNPs):**
The 15 cryopreserved salivary aptamers obtained from BPB were brought to room temperature and diluted to the required concentration (2nM) using the folding buffer solution as described in the BPB best-practices documentation provided for the aptamers. The aptamers were then folded by incubating at 95°C for 5min and kept at room temperature for another 15min. Apt-AuNPs conjugates were prepared by mixing the gold nanoparticle solution and folded aptamers at a 1:1 ratio. Different concentrations of soluble salivary protein extract and recombinant D7 protein in PBS and SFS were used to first screen each of the new Apt-AuNPs. Bovine serum albumin
(BSA, Sigma-Aldrich, St. Louis, MO) was used a negative control protein. The best performers in the screening assay were then selected for further assessment to determine the spectroscopic and visible limits of detection. Aggregation/response of the Apt-AuNPs was evaluated using UV/Vis spectroscopy (Perkin Elmer Lmabda-750S) and visual inspection.

**Results and Discussion**

*Isolation, Purification and Characterization of Mosquito Saliva Proteins:*

Both methods of collecting salivary proteins were successful. Images of both detailing both methods are shown in Figures 1 and 2; silver-stained protein gels characterizing the obtained proteins are shown in Figure 3.

![Figure 1](image1.png)  
**Figure 1.** *Ae. aegypti* salivating into capillary tubes with immersion

Over 12µg of protein was recovered from the directly-collected saliva from thousands of mosquitoes and ~75µg of soluble protein (both without detergents) was retrievable from salivary gland extractions from 200 mosquitoes. Given that each mosquito maximal excretes less than 1µL of saliva containing no more than ~20ng\textsuperscript{15,16}, collections from thousands of mosquitoes were required to collect

![Figure 2](image2.png)  
**Figure 2.** Dissection and isolation of female *Ae. aegypti* salivary glands. A) Female mosquito with legs and wings removed. B) Separated head with salivary glands attached (white arrowhead). C) Intact, isolated salivary glands stained with eosin to facilitate visualization.

![Figure 3](image3.png)  
**Figure 3.** Silver-stained protein gels characterizing and comparing *Ae. aegypti* saliva collected in Mexico (S MX) and salivary gland (SG) extracts at the USDA-ARS CMAVE.
the 10’s of μgs required for the aptamer development. We therefore favored isolating salivary proteins from salivary glands as much as possible to meet the project needs.

**Salivary Apt-AuNPs Synthesis, Characterization and Function:**

The Apt-AuNPs created from salivary aptamers received from BPB, 1-9 (developed against the saliva collected by the Machain-Williams lab) and 10-15 (developed against soluble salivary gland extracts), were all responsive to 1ng of recombinant D7 salivary protein in PBS buffer. The responses in this screening assay are evidenced by the solution color change and an intense shift in the peak of the UV/Vis spectrum in the presence of D7 protein (Fig. 4). Apt-AuNPs created using aptamer 1 showed the highest peak intensity at ~640 nm followed by Apt-AuNPs created with aptamers 2 and 3 as can be seen from Figure 4(a). Apt-AuNPs with aptamer 15 showed maximum change after the 30min incubation time among the aptamers developed against the salivary gland extract target (Fig. 4(b)). Therefore, Apt-AuNPs with aptamers 1-3 and 15 were selected for further studies.

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**Figure 4.** UV/Vis spectra of AuNPs-aptamer in presence of D7 protein (1ng) in PBS buffer after 30min incubation. An intense agglomeration was observed in all the Apt-AuNPs solutions as compared to the control (BSA) sample. (a) Shows agglomeration in Apt-AuNPs with aptamers 1-9 whereas (b) indicates agglomeration in Apt-AuNPs with aptamers 10-15. It can be seen that all the Apt-AuNPs solutions were aggregated. (c) Image showing the visible color change in all the aptamer solutions from red to gray. Note that no color change or peak shift in the UV/Vis spectrum was observed in the BSA control.
The results in SFS against recombinant D7 protein of the four (4) Apt-AuNPs selected from the screening assay are shown in Figure 5. Unlike the response observed for 1ng of D7 in PBS, a peak shift in the UV/Vis spectra required the addition of at least 10ng of D7 (Fig. 5a-d) and a visible color change started to occur after 40ng of D7 were added (Fig. 5e). Increasing the concentration of D7 to 40ng produced an immediate color and peak shift in the UV/Vis spectrum (Fig. 6a) that intensified after a 16h incubation (Fig. 6b). The BSA negative controls showed no peak shift in the UV/Vis spectra nor a change in the solution colors with increasing D7 concentrations.

![Figure 5](image_url)

**Figure 5.** UV/Vis spectra of Apt-AuNPs conjugated with aptamers 1, 2, 3, or 15 in presence of recombinant D7 protein in SFS. (a) Spectra of all Apt-AuNPs solutions showing a peak at ~640nm due to aggregation in presence of 20ng of D7 protein. (b) Spectra showing small peaks at ~640 due to the addition of 10ng of D7 protein; maximum aggregation occurred with Apt-AuNPs with aptamer 15 followed by Apt-AuNPs with aptamer 1. (c) and (d) Spectra showing no peak at ~640nm in the presence of D7 (5 ng, 2 ng) respectively. No peak shift in the spectra was observed for BSA controls. (e) Image of the Apt-AuNPs solutions in presence of D7 or BSA protein (2-20 ng). No immediate color change was observed in any of the solutions.

Preliminary testing of Apt-AuNPs with aptamers with either aptamers 1 or 15 tested against extracted soluble salivary proteins in the SFS did not exhibit the same response as when tested against D7 alone (Fig. 7). No change in visible solution color was observed immediately after adding the extracted proteins (2 or 3µg) for either of Apt-AuNPs, however an obvious change
and aggregation/precipitation occurred after 48h. BSA negative controls showed no color change immediately for either Apt-AuNPs; a slight shift was observed in Apt-AuNPs with aptamer 15.

**Figure 6:** Images of the visible color change and respective UV/Vis spectra for Apt-AuNPs with aptamers 1, 2, 3, or 15 against 40ng of D7 or BSA protein in SFS. (a) Immediate color change and (b) after 16h incubation; aggregation of the Apt-AuNPs in solution increased over time and the color change intensified. BSA negative controls showed no change in color or peak shift in the UV/Vis spectra even after several hours.

**Figure 7:** Images of visible color change in solutions of Apt-AuNPs with aptamers 1 or 15 against soluble salivary protein extract in SFS (2, 3µg). Intense color changes were seen for both 2µg and 3µg additions of soluble salivary proteins after 48h in both solutions of Apt-AuNPs. No color change was seen for BSA negative controls immediately and only slightly for Apt-AuNPs with aptamer 15 after 48h.
The results of the present study are extremely promising and represent the first development of aptasensor tools capable of specifically detecting *Ae. aegypti* salivary proteins (D7). The empirical visible limit of detection (LOD) for D7 protein of 1ng in PBS and 40ng in SFS—without the application of a developer solution (NaCl)—supports the contention that a colorimetric aptamer-based membrane sensor can be created capable of detecting female *Ae. aegypti* mosquito via saliva expectorated during sugar-feeding. More research must be conducted however into improving the LOD and aptasensor response to whole saliva in our SFS to realize such a field surveillance sensor. Our next experiments will therefore focus on exploring different aptamer and AuNP concentrations and ratios, creating multifunctional Apt-AuNPs, addition of salt developer solutions, more testing with directly collected saliva and soluble salivary protein extracts and incorporation of the entire system in a paper/membrane format.
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