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Optimized Gold Nanoparticle Aptamer-Based Sensor To Detect Boar Taint

Nana Frimpong
Institute of Biochemistry, Carleton University
Ottawa, Ontario
Canada, K1S 5B6.

Faculty Supervisor: Dr. Maria C. DeRosa*
Mentors: Dr. Velu Ranganathan* and Josh Callahan*

Abstract

Boar taint is an unpleasant natural aroma that can be detected when cooking or eating porcine samples with high levels of skatole or androstenone. To control the level of boar taint, farmers resort to early castration and immunological hormone suppression procedures in pigs. These control methods are expensive and require high technological expertise. Therefore, a fast, cost effective and simple alternative is needed to help farmers and butchers keep boar taint out of consumer pork. Aptamers are single stranded oligonucleotides that can be used to detect small molecules with high affinity and specificity. An aptamer-based sensor using gold nanoparticles with easily measurable optical properties has been already developed to detect skatole or androstenone in consumer pork. The potential of the gold nanoparticles to aggregate in a saline medium is improved with the binding of the aptamer to a target molecule. However, the limits of detection of the sensor are unknown. In the present study, we successfully optimized the existing aptamer-based method using the gold nanoparticle-binding assay and Uv-vis analysis to enhance reproducibility, sensitivity and cost effectiveness of the sensor. DNA aptamers (SKT 1 and SKT 2) were used in the gold nanoparticle aptamer-based sensor trials because they bind with high affinity and selectivity to the skatole and androstenone targets. The optimized gold nanoparticle-binding assay revealed that each component (DNA, sodium chloride and citrate) of the assay greatly affected the performance of the sensor. The nanoparticle-based sensor produced a colour change from pink to dark purple when skatole or androstenone targets were added after salt-induced aggregation. This can be spectophotometrically detected at 660 nm. These findings support the applicability of the optimized sensor for practical onsite use by farmers and butchers. The developed sensor has various scientific applications but further work is required to deploy and ensure successful use worldwide.

Key words: Boar taint, Aptamer, Gold nanoparticles.

1. Introduction

1.1 Boar Taint

Boar taint is an unpleasant aroma that can be evidenced in cooking or eating pork with high levels of skatole and androstenone.[1] These two compounds are lipophilic so they can be deposited in the adipose tissue of growing pigs and their volatile nature also enhances detection when cooking pork samples with high levels of these compounds.[2] Boar taint is more likely to occur in male than in female pigs and it is moderately heritable.[3] Pigs suspected to have boar taint have to undergo immunological suppression procedures, slaughtered at an early age or castrated to eliminate boar taint.[4] However, pigs that do not have boar taint will not have to undergo these expensive and complicated procedures with the advent of enhanced boar taint detection.

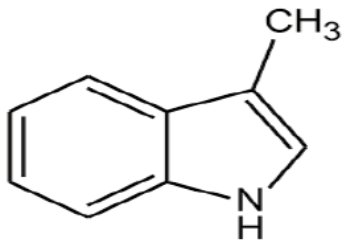

Figure 1: Chemical structure of the large intestine product , **Skatole**.[1]

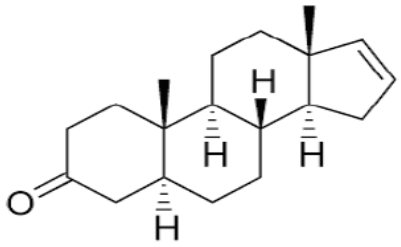

Figure 2: Chemical structure of the pheromonal steroid, **Androstenone**.[7]

Castration involves making a cut on either side of the pig scrotum, pulling out the testicles and cutting them off or out.[5] It takes about 3-6 weeks for the androstenone compound causing boar taint in male pigs to disappear completely after castration. Boar taint when present makes pig meat smell and taste awful but it is in only about 20 % of male pigs.[6] This means that 80 % of male pigs are being castrated unnecessarily.

Androstenone levels are mostly determined by genetic factors and stages of puberty, whereas skatole levels are controlled by nutritional or environmental factors in addition to genetic background or hormonal status of the pigs.[7] Some breeds of pigs have higher levels of boar taint than others. Durocs tend to have higher levels than Yorkshire and Landrace breeds.[8] Castration takes time for the farmer and if the cut gets infected, this would require medical treatment, set back in weight gain or even death for the pig. Pig farmers have many options when picking potential breeders from a pool. Unfortunately, castration performed as a control measure for the reduction of boar taint levels in pigs closes that door early and limits their breeding options. Thus, a fast, sensitive and selective detection method for skatole and androstenone is of great need for on the spot screening of live pigs via biopsy or carcass at processing.[7,8]

1.2 Boar Taint Detection

Many methods have been developed to detect boar taint, which include analytic methods like High Performance Liquid Chromatography (HPLC) or gas chromatography. Sensory methods involve manual smelling by a trained sensory panel.[9] The methods for analyses in both of these categories have proven to be very selective but very unreliable since they are performed with no standardized protocol.[10] Thus there is the need to develop a standardized protocol to detect boar taint where porcine samples are analyzed and quantified quickly with high reproducibility.

Optical biosensors are transducers which involve the determination of changes in light absorption between the reactants and products of a reaction and could also involve the measuring of light output by a luminescent process.[11] When an analyte solution with a molecule target of interest comes into contact with the biological recognition element in an optical biosensor, the response from the recognition element is converted to a signal that can be seen with the application of colorimetry.[12] Optical biosensor assays are more likely to be an improved way to detect if a porcine sample has boar taint because of their ease of detection and cost efficiency.[13]

1.3 Aptamers as Biological Recognition Elements in Optical Biosensors

Aptamers are chemically synthesized single stranded deoxyribonucleic acid (ssDNA) or single stranded ribonucleic acid (ssRNA) that bind to molecule targets with a high affinity and a high specificity.[14] They are selected using an in vitro process known as Systematic Evolution of Ligands by EXponential Enrichment (SELEX) based on their affinity for a target molecule which can be manipulated to a high degree.[15] The recognition of small molecules by aptamers is governed by hydrogen bonding interactions, steric effects, electrostatic interactions, Van der waals forces, stacking of flat parts of a functional group and tiny conformational changes.[16] Aptamer structure and binding domains are predicted based on the assumption that they have a variety of three dimensional conformations ascribed to their structures which include; stem loops, G-quadruplexes and double-stranded helical segments.[17]

Aptamers are able to detect a variety of molecules both large and small since they are synthesized chemically.[18] The range of detection is from organic molecules, ions, toxins, complex macromolecule, drugs to even whole cells.[19] This enables aptamers to be used in optical biosensors, in the agricultural industry to guide smart fertilizers to plant roots,

to guide drugs to specific targets in the body and used as therapeutic tools in medicine, and many other applications.[20] The specificity and characteristics of aptamers is determined by their tertiary sequence.[21]

Aptamers are great biological recognition elements for small molecule targets since they are cost effective as recognition elements in sensor applications, have a high thermal stability with resistance to temperatures over 80 ℃, can easily be modified at precise locations with reporter molecules without affecting binding affinity, can be stored for extended periods of time and have a low batch-to batch variability.[22]

Aptamers are generated from large random libraries of chemically synthesized oligonucleotides of a specific size through a process known as SELEX.[23] In the selection process, a column matrix of agarose, sepharose or magnetic beads is used depending on the target molecule.[24] A typical library contains a randomized region of nucleotides flanked by two constant regions for polymerase chain reaction (PCR) amplification. The large random library is incubated in the column matrix and exposed to a single target. The column is rinsed to remove any unbound target molecules and the bound aptamers are eluted out under conditions of high salt, temperature and urea to disrupt the target-aptamer complex.[24]

The eluted aptamers are then PCR amplified and enriched to increase their affinity and selectivity for the target molecule before the next SELEX round. With each SELEX round, the strictness with which the Watson – Crick base-pairing is required under specified conditions of temperature, pH and salt concentration is increased.[23] This is such that all bases of one polynucleotide are paired up with complementary bases on the other until the only remaining aptamers in the pool are highly specific for and bind with high affinity to the target.[23] Once multiple rounds of SELEX are completed, the DNA sequences are usually identified by conventional cloning and sequencing.

Two aptamers were selected for study based on a high affinity and selectivity for skatole and androstenone through a process known as capture SELEX. The capture SELEX process allows for the selection of ssDNA aptamers for solute targets using streptavidin coated magnetic beads that are conjugated with biotinylated capture oligonucleotides rather than a stationary column.[24] The DNA pool of aptamers have a complementary docking sequence incorporated into their random region which enables hybridization to a complementary oligonucleotide fixed on the magnetic beads.[24]

The aptamers of the pool which show high affinity to the target and a secondary structure disrupting the aptamers capacity to bind to the docking sequence are released from the beads by binding to the target during the capture SELEX process.[24] The aptamers from those binding complexes are amplified, purified and remounted through the docking sequence to the magnetic beads at the beginning of the next selection round. The two aptamers selected for study through capture SELEX were SKT 1 and SKT 2 because there was optimal separation of the aptamers in colloidal solution and preferential binding of the target molecule after it was introduced with observable colour change from pink to purple after the addition of NaCL . The evidence of this optimal separation was observed in a significant absorbance shift from 524 nm to 660 nm in comparison to the other aptamers of the pool which did not have much of a significant shift.[23]

Consumer fat dissolved in ethanol and water was used for the final round of selection of skatole and for the final counter selection of androstenone. The aptamer, SKT 1 is a 91 base long, normal sequence aptamer and the SKT 2 aptamer is an 80 base long, short sequence aptamer generated by base deletions during SELEX. Their K_d values have not yet been determined. The final enrichment of the pool was sequenced using MiSeq and HiSeq Illumina technologies.[25]

1.4 Gold Nanoparticles (AuNP) As a Reporting Probe In Optical Biosensors.

Colorimetry used in an agricultural farm setting to detect boar taint in pork samples as a regular drill could prove to be even more complicated when it is incorporated into a procedure that is cumbersome or produces unreliable results. Aptamers can also be paired up with nanoparticles which are reporter probes with large surface areas, varying sizes and shapes with interesting optical properties which allow for easy detection of biological molecules at tunable wavelengths.[26] Gold nanoparticles (AuNP) are sub-micrometer suspensions of gold in a fluid which could be paired up with aptamers in an assay to detect levels of a specific biological target in solution.[27] AuNP in an assay together with aptamers will be an excellent pair since they absorb and scatter light with a great efficiency at tunable wavelengths dependent on size.[28]

The conduction band electrons on the metal surface of the AuNP undergo a group oscillation when hit by light at specific wavelengths through a phenomenon known as surface plasmon resonance (SPR) when the gold is used on a nano-scale.[29] They have an intense red or pink color when particles are small (3.3 nm) and an intense blue color when its particles are aggregated (330 nm) by salt induction which changes its optical properties and SPR.[29] In addition, AuNP are stable, non-toxic, conductive, and they allow the adsorption of DNA or RNA sequences to their surface

which modifies their surface. The visual aggregation of AuNP by salt induction is a useful property that can be used to determine target levels in solution when aptamers are incorporated.

Citrate-capped AuNP that were used in this study were synthesized by the Turkevich method which involves the growth of AuNP by citrate reduction.[30] AuNP exist in a dispersed state due to the negatively charged citrate molecules present in solution which act as capping agents.[31] The aptamers can be adsorbed electrostatically onto the surface of the citrate capped AuNP and this adsorption protects the AuNP from salt induced aggregation and is visualized as a pink colour. When the specific target is present in solution, the aptamer will preferentially bind the target molecule and leave the AuNP surface.[32]

The aggregation of the nanoparticles can result from changing the temperature, pH and ionic strength of the solution which caused the double-layered particles to come close together causing the interparticle distance to be smaller than the average diameter of the particle supported by the DLVO theory.[33] The theory combines the effects of Van der waals attractive forces and the electrostatic repulsion due to the imaginary double-layer of counter ions to explain the charged surface interactive forces as well as the aggregation of aqueous dispersions quantitatively.[33]

An aggregated state results in a change of the interparticle plasmon coupling as the refractive index of medium around AuNP is reducing, which produces a huge blue shift in the absorption band from 524 nm to 660 nm in the visible region of the electromagnetic spectrum .[34] The change in color from pink or red to purple or blue confirms the presence of target in solution in a quantitative manner. A high AuNP concentration gives a rich colour and a greater absorbance signal for analysis.[34] DNA aptamer concentration has to be matched accordingly with AuNP to ensure proper surface coverage and UV-vis spectrophotometry can be used as a simple and reliable way to quantify the colour change and monitor the stability of nanoparticle solutions.[35]

UV-vis spectrophotometry is the absorption spectrophotometry in the ultraviolet –visible spectral region and depends on the detected colour of the chemicals involved. Transmission electron microscopy (TEM) was another way to visualize the change from non-aggregated to aggregated states of the gold nano-particles.[36]

2. Methods

2.1 Materials and Instruments

Skatole and Androstenone stock samples used in the AuNP assay were obtained from the Canadian Center for Swine Improvement Inc (Ottawa, ON, CA). All solutions were prepared with Millipore Milli-Q deionized water. All micro centrifugation tubes, salts, reagents, buffer solutions were purchased from Sigma- Aldrich. Unmodified aptamer stocks were obtained from Integrated DNA Technologies (IDT). UV-vis absorption spectra were obtained using a Varian Cary 300 Bio UV-vis Spectrophotometer with a 1 cm path length fused quartz cuvette.

2.2 DNA Quantification[35]

The concentration of DNA in the different samples was calculated by taking the absorbance of the samples at 260 nm by UV-vis spectrophotometry. Unmodified DNA aptamer stocks were obtained from Integrated DNA Technologies (IDT).

2.3 Gold Nanoparticle (AuNP) Binding Assay[37]

2.3.1 androstenone and skatole stock solution preparation.

The stock solutions for skatole and androstenone were prepared by serial dilutions to concentrations ranging from 0 M , 1.0×10^{-4} M to 1.0×10^{-13} M with 100 μL of 1.0×10^{-4} M skatole or androstenone in 900 μL water. All unmodified aptamers were purchased from IDT. Each stock was diluted to 10 μM before use.

2.3.2 Standard AuNP Trial Setup

Aptamers can be adsorbed electrostatically onto the surface of citrate-capped AuNPs and this adsorption protects the AuNPs from salt-induced aggregation. When the specific target molecule is present in solution, the aptamer will preferentially bind the target molecule and leave the AuNP surface.The addition of salt to the solution with target,

AuNP and aptamer will induce aggregation this confirms the presence of the skatole or androstenone target. High AuNP concentration gives a rich colour and a greater absorbance signal and DNA concentration should be matched accordingly.

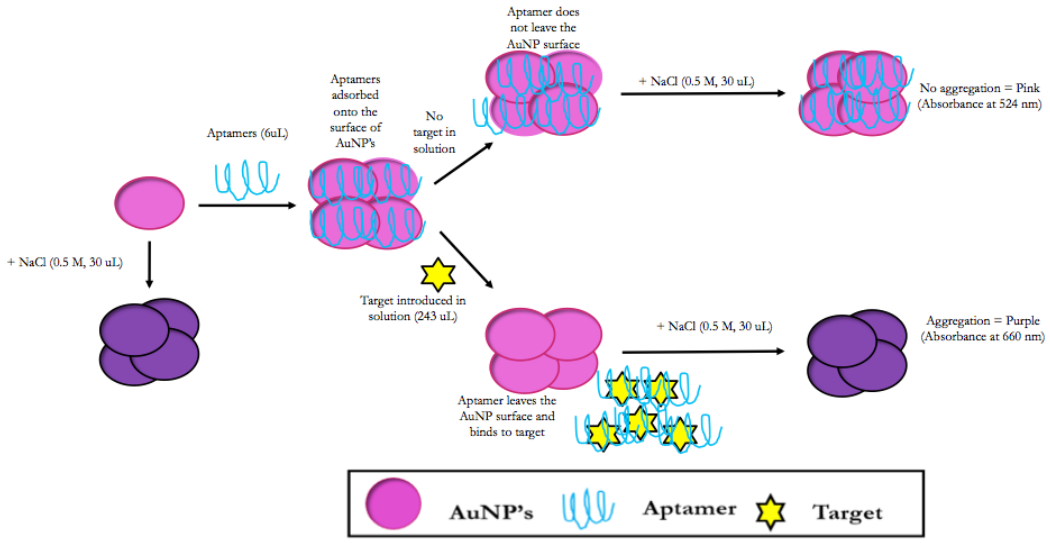

Figure 3: Flowchart visually depicting what happens molecularly during the salt-induced AuNP aggregation assay with and without target in solution.

3. Results and Discussion

Boar taint can be a huge problem for agricultural farmers since they have to select the best pool of animals for breeding and prevent affected animals from breeding so their consumer target is satisfied with their resulting porcine products.[6] Professional smellers detect boar taint currently and this is not always accurate.[10] The main goal of this study was to develop a sensor to rapidly detect levels of boar taint onsite while enhancing the reproducibility, sensitivity and cost of the sensor.

The goal was achieved by using Gold nanoparticles as a chemical reporting probe in an assay to qualitatively detect the levels of skatole and androstenone in different concentrations with DNA aptamers as recognition elements.[37] This will improve the stressful and inaccurate procedure of detecting boar taint so that pig samples with boar taint can be eliminated before getting on the market so consumers are satisfied.

3.1 Gold Nanoparticle (AuNP) Binding Assay with Targets

Single stranded DNA (ssDNA) aptamers were incubated with citrate-capped AuNP for 30 minutes. The ssDNA is influenced by electrostatic interactions when it adsorbs to the surface of the AuNP.[16] The adsorption of the aptamers to the surface of the AuNP is possible due to the negatively charged backbone of the ssDNA aptamers. ssDNA aptamers could have an A , T, G or C base.[38] An A base will be able to bind favourably to the AuNP surface since it has a negatively charged nitrogen on its heterocyclic imidazole ring and an amine on its purine ring.[38] The negatively charged nitrogen atoms will be attracted to the positively charged thin outer sodium atom layer and not the negatively charged citrate atom thin inner layer and of the AuNP.[38] The negative charge displaces the citrate and the aptamers takes on its place as a surfactant.[38]

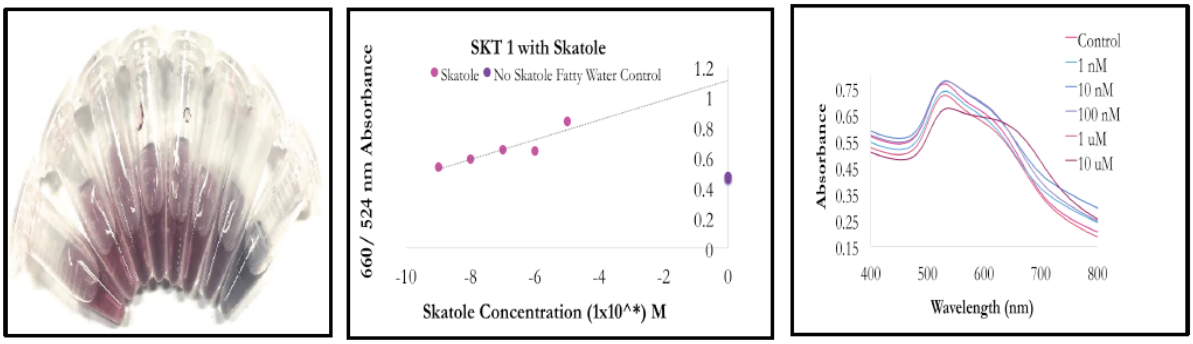

Figure 4: Image from colorimetric AuNP assay (left) , UV-Vis plot of different Skatole Absorbance's (0.1nM,10nM,100nM,1uM and 10uM) against wavelength (nm) generated by excel (middle) and UV-Vis plots of 660/524 nm Absorbance ratio against Skatole Concentrations generated by excel (right) showing the effect of having no Skatole in the AuNP sensor and projecting the salt induced aggregation trend which shows an increase in Absorbance as Concentration increases.

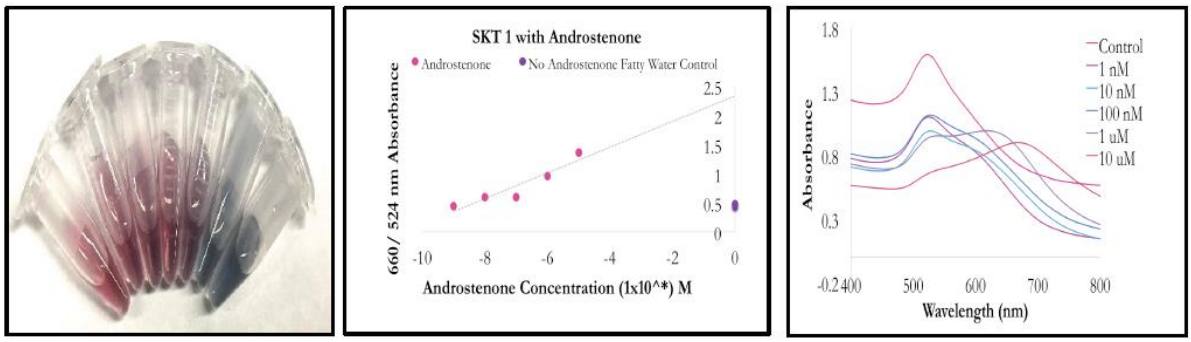

Figure 5: Image from the colorimetric AuNP assay (left) , UV-Vis plot of different Androstenone Absorbance's (0.1nM,10nM,100nM,1uM and 10uM) against wavelength (nm) generated by excel (middle) and UV-Vis plots of 660/524 nm Absorbance ratio against Skatole Concentration generated by excel (right) showing the effect of having no Skatole in the sensor and projecting the salt induced aggregation trend which shows an increase in Absorbance as Concentration increases.

Once the aptamer is successfully adsorbed onto the AuNP surface, there is an even distribution and separation of the aptamer strands in solution during the incubation period. When the skatole or androstenone target molecule is added, binding of the ssDNA aptamers to the target will be favoured over being bound to the AuNP surface because of the high affinity that exists between them.[23,32] The higher the concentration of target in solution, the more aptamers will leave the surface of AuNP to preferentially bind. The lower the concentration of target in solution, the less aptamers will leave the surface of AuNP to preferentially bind.[23,32]

The stability of the colloidal solution with AuNP, ssDNA aptamer and target is then affected after the addition of 0.7 M sodium chloride (NaCl). The salt disrupts the citrate double- layer surfactant and shrinks it to the point where all the other AuNP in solution come together and aggregate.[33] An energy barrier exists between each citrate-capped AuNP molecule which is balanced by electrostatic repulsion and Van der waals attraction.[33]

Electrostatic repulsion is high when the two AuNP approach each other and their double layers begin to interfere.[33] An increase or decrease in the ionic or pH environment or the addition of surfactant affects the stability between the AuNP and the reduction of the double layer.[33] The optimal amount of salt results in the destabilisation of the AuNP and causes the observable colour change from red or pink to blue or purple.[29]

The two selected strong aptamers were tested in a Gold nanoparticle-binding assay in the presence of skatole or androstenone at concentrations ranging from 1.0×10^{-4} M to 1.0×10^{-13} M . Their absorbance was measured upon colour change and of all the aptamers candidates, SKT 1 and SKT2, showed significant colour change with their absorbance spectra revealing a shift in wavelength from 520 nm to 660 nm. Many different factors (DNA levels, Salt levels, Citrate levels and Target levels) come together to ensure that the sensor works the best. Optimizing each of

these levels will ensure that the sensor produces an increase in aggregation of the Gold nanoparticles with an increase in target concentration.

3.2 Salt Induced Aggregation with Negative Controls

Salt induced aggregation UV-vis results with SKT 1 Aptamer , Tryptophan and Indole negative controls tested the specificity of the bound aptamer. Skatole and Indole have very similar structures, differing by only a methyl group[1,7] so there is a possibility that indole may exsist in the target skatole solutions. Tryptophan is the metabolic precursor to Skatole and has structural similarities to Skatole thus the need to control for it. No significant colour change was produced in both cases after salt induction as expected for any of the aptamers and no significant shift of the absorbance spectra as shown below.

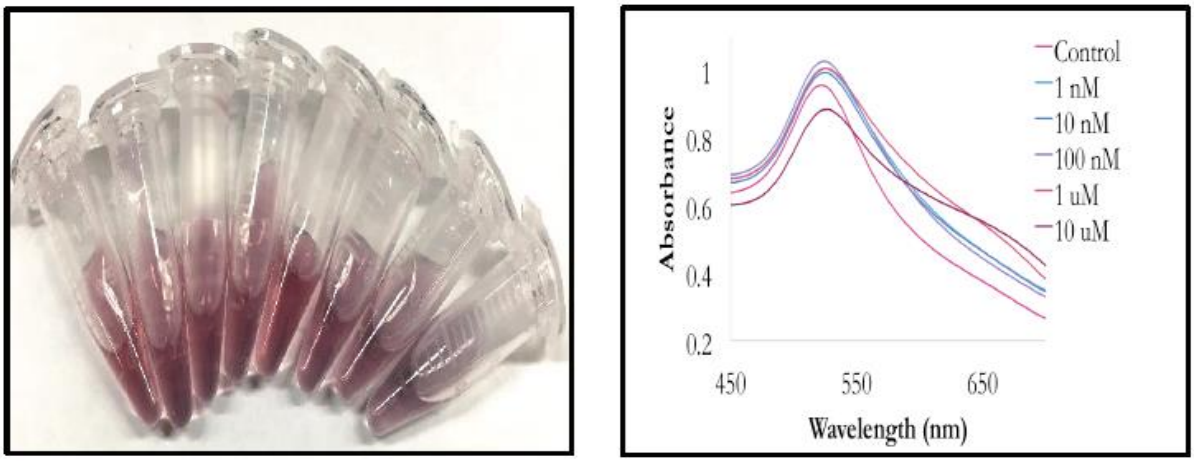

Figure 6: Image from the AuNP colorimetric assay results (left) and UV-Vis plot of different Indole Absorbance's against wavelength (right) showing the effect of having no Indole in the AuNP sensor with minimal colour change after salt induction which means as concentration increases, absorbance remains similar.

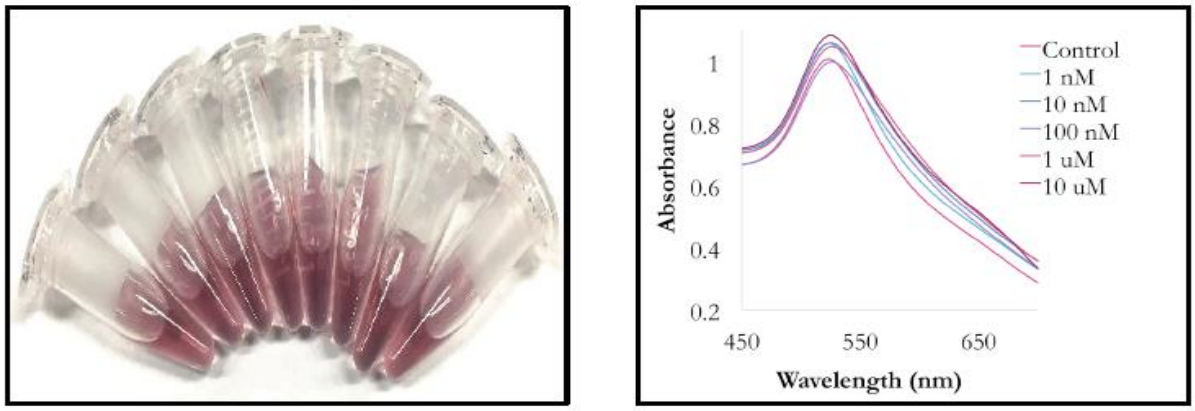

Figure 7: Image from the AuNP colorimetric assay (left) and UV-Vis plot of different Tryptophan Absorbance's against wavelength showing the effect of having no Androstenone in the AuNP sensor (left) with no colour change after salt induction which means as concentration increases, absorbance remains similar.

3.3 Conclusion and Future Direction

From the study , the AuNP Aptamer based Assay was successfully optimized with known amounts of NaCL, DNA and Target for each trial. There is the need to further develop the gold nanoparticle assay and synthesis method however to accommodate interfering variables and produce consistent results. There is the need to also undertake

dissociation constant studies to better understand Aptamer target interactions and finally to develop a lateral flow assay device to make the sensor applicable for practical use and provide rapid onsite detection for agricultural farmers.

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