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Locked Nucleic Acid Aptamer and 10 nm Gold Nanoparticles Increases the Sensitivity of a Prion Protein Detection Assay

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I. Abstract

Chronic Wasting Disease (CWD) is a prion-caused ailment that primarily affects cervids and causes fatal neurodegenerative disorder. To contain and surveille CWD, detection in live animals is imperative. Antemortem detection of CWD is the overall goal of the research. Specifically, the aim of this project is to design an immunoassay with improved sensitivity for detection. As CWD is an infectious prion requiring a government approved laboratory, normal prion protein (PrP^{C}) was chosen as the model protein to test and optimize this assay. Employing an enriching, magnetic bead-based ELONA (enzyme linked oligonucleotide assay), a PrP-specifc aptamer – 17OAp1-24 – served as the detection probe. Furthermore, incorporation of locked nucleic acids (LNA) in the aptamer further increased the sensitivity of the assay. Finally, functionalizing 10nm diameter gold nanoparticles with aptamer and biotin significantly improved the signal generated in the assay. It is expected that further testing with different gold particle sizes will affect sensitivity as well. These results suggest that this assay could provide a promising avenue for CWD detection in antemortem samples.

II. Introduction

II.a Chronic Wasting Disease: significance and background

Chronic Wasting Disease (CWD) is a progressive, fatal disease that affects the brain, spinal cord, and many other tissues of captive and free-ranging cervids such as deer, elk, and moose. CWD belongs to a family of diseases called prion diseases or transmissible spongiform encephalopathies (TSEs). The infection is caused by abnormally folded proteins called prions. Prions are found in most tissues in the body and can be shed in urine, saliva, seminal fluid, and feces. They are nearly indestructible, and normal environmental processes such as sunlight, freezing, and desiccation do not break down prions once deposited into the environment. CWD is transmitted via oral route and most likely occurs by direct contact with infected animals, or through indirect contact with environmental deposits (Haley 2015, Benestad 2018, Osterholm 2019).

First identified in 1967 in a research facility in northern Colorado, CWD has been found in the free-ranging cervids spread across 26 states, 4 Canadian provinces, and Sweden, Norway, Finland, and South Korea. The number of free-ranging cervids affected by CWD has been increasing in the US (WDON Resources 2017), and the disease is contributing to the decline of the cervid population (DeVivo 2017), thereby posing a significant threat to the conservation of the animal species. In addition, the spread of CWD in the free-ranging animals discourages big game-hunting which contributes $26B to the US economy (US DOI 2016). In 2004, Bishop's study estimated the annual economic impact of CWD to the hunters in Wisconsin to be over $70M, and $5M to the state (Bishop 2004). The disease was identified not only in the free-ranging animals but also in the captive cervid facilities with a high infection rate (79%) (Keane 2004). In 2014, Anderson et al. estimated that the impact of CWD on the US captive cervid industry to be $230M (Anderson 2014).

II.b Bead-based enrichment of target molecules

Amplifying the signal of immunoassays for the strongest detection has been a point of interest in research. Multiple methods have been put forth. The first being that of magnetic beads. As Wadhwa and colleagues point out with their study on *Mycobacterium avium* the usage of magnetic beads when coated with the antigen for the bacterium and allowed to incubate with secondary antibody proved to be a plausible avenue for the creation of a serodiagnosis method (Wadhwa 2012). A similar concept was used by Denkers group, leveraging CWD prion affinity to metal to enrich from tissue homogenates using iron oxide magnetic beads (Denkers 2016).

II.c Aptamers: advantages and use with prion detection

An aptamer has been defined as "nucleic acid molecules that mimic antibodies by folding into complex 3D shapes that bind to specific targets". These molecules are advantageous over antibodies for a variety of reasons. Namely, the generation process of aptamers is good, as the

production cost and batch-to-batch variability is low. They are also produced chemically, which ensures bacterial and viral contamination is limited. Aptamers also have reversible folding properties, meaning that when unfolded, their function is not affected. Shipping antibodies requires specific transport conditions, such as cold temperatures, and the unfolding ability of aptamers allows the overcoming of this. Moreover, aptamers can be made to bind a wide variety of chemical and biological targets from small to whole cells (Dunn 2017). Overall, aptamers allow more specificity.

In 2011, Wang et al. successfully developed aptamers against PrP (prion protein), normal and abnormal forms. They predicted that the interaction region is that of the N-terminal and a nucleic acid binding site on PrP. They found that aptamer 17OAp1-24 specifically bound proteinase K-digested PrP (Wang, 2011). Prion protein was chosen as the model protein to test in our system, hence the choice of this specific aptamer.

The conformation of aptamers can be set as well by use of Locked Nucleic Acid (LNA), which are types of nucleotides where "the ribose sugar [are] constrained in the 3'-endo conformation via a bridge between the 2' and 4' carbon atoms" (Roberts, 2020). The modification is beneficial as it enhances the stability and affinity of the molecules for its target.

II.d Enzyme Linked Oligonucleotide Assay: immunoassay with oligonucleotides

A test in which these aptamers can be used for detection is called an ELONA (Enzyme Linked Oligonucleotide Assay). Like many other immunoassays, this approach employs antibodies to capture the ligand of interest. However, instead of a detection antibody, the aptamer serves as the probe. The aptamer can be further modified by linking one of the ends to biotin, for example, and take advantage of frequently used streptavidin-based complexes. This allows it to be used in conjunction with a variety of colorimetric, fluorescence, or electrochemical detection methods.

II.e Gold nanoparticles in detection of analytes

Gold nanoparticles (AuNP) offer a promising avenue to increasing sensitivity. The characteristic of these particles provides numerous benefits: they can be made easily and are highly stable, have unique optoelectronic properties that allow for detection with light/color change, have high surface to volume ratio and compatibility when using the correct ligand, can be readily tuned with suitable molecules for selective binding and detection of small targets and improves selectivity. Their properties that allow colorimetric sensing are of specific interest, as in conjunction with an aptamer-based method, the AuNPs have allowed for detecting small molecules through colorimetric detection, along with proteins that have been associated with diseases (Saha, 2012).

II.f Project aims

The purpose of this study is to design an assay with improved sensitivity for the detection of PrP using a locked nucleic acid version of the 17OAP1-24 aptamer, magnetic beads, and gold nanoparticles (**Figure 1**). This test would bridge the gap between conventional and amplification-based testing methods, which all suffer from several disadvantages (**Table 1**).

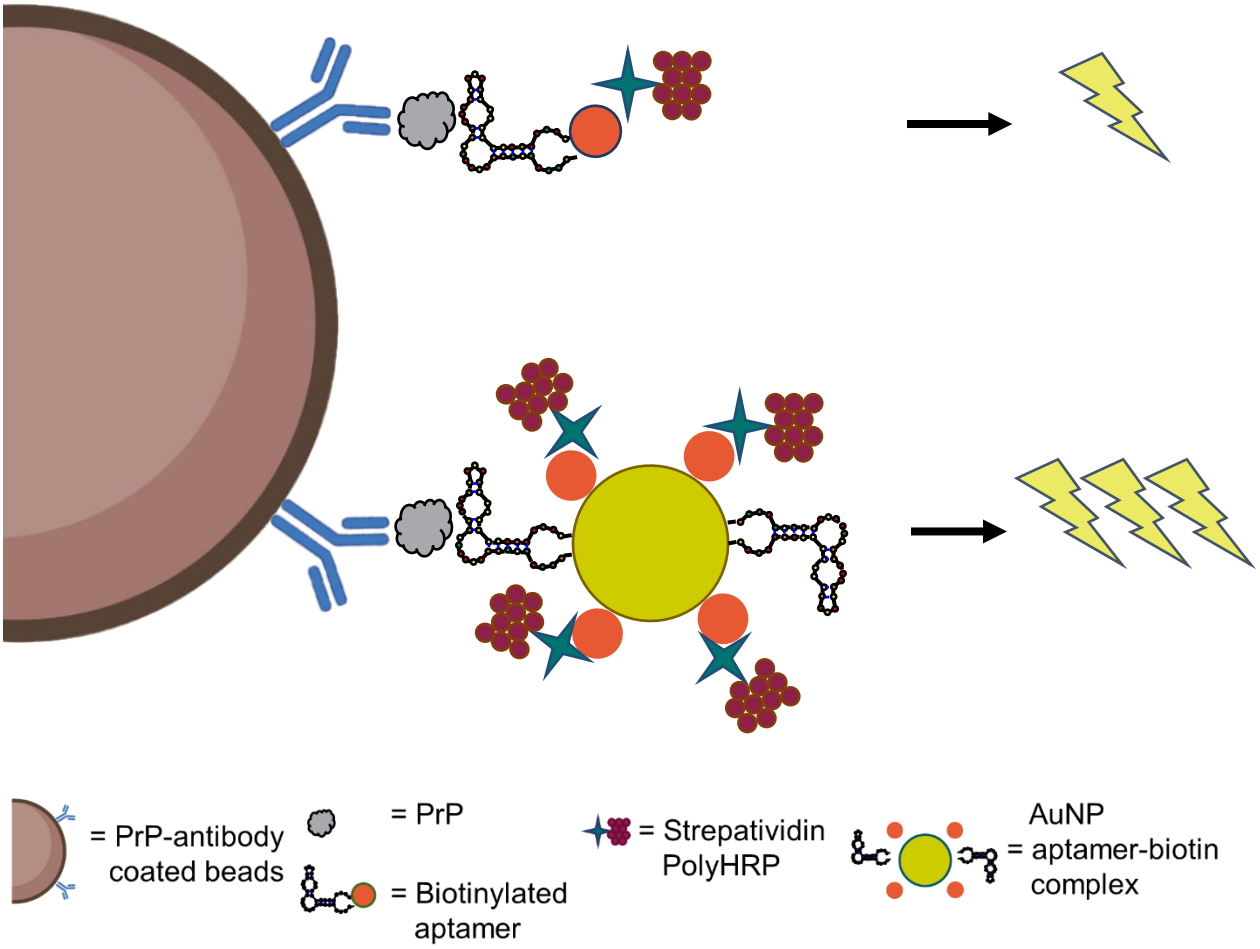

Figure 1 – Overview of assay design. An overview of the detection method using prion protein (PrP)-specific antibodies coupled to magnetic beads, aptamer, and gold nanoparticles. The signal was quantified by measuring optical density at 655 nm (OD655).

	Tests	+ Pros	- Cons
Conventional Methods	• ELISA • WB • IHC	• Relatively fast test	• Needs post-mortem tissue • Low sensitivity
Amplification Methods	• PMCA • RT-QuIC	• Improved sensitivity	• Long assay time (>3 days) • Susceptible to false-positives • Requires prion substrate

Table 1 – Comparison of current detection methods highlighting pros and cons of each approach.

III. Methods

III.a Reagents

The following reagents were purchased from Fisher Scientific, with indicated catalog number: 10X Phosphate Buffered Saline pH 7.4 (#AAJ62036K2). Gold nanoparticles 10 nm supplied in 0.1mg/mL sodium citrate with stabilizer, OD1, 520nm absorption (# J67188AE). TE Buffer, Tris-EDTA (TE buffer), 1X Solution, pH 8.0, Molecular Biology (#BP2473100). Thiolated PEG Biotin, 400Da PEG (#PG2-BNTH-400). Immobilized TCEP Disulfide Reducing Gel (# 77712). NHS-activated magnetic particles, 10 um (#88826). Thermo Scientific SuperBlock (PBS) Blocking Buffer (#PI37515). Fitzgerald Industries International Streptavidin Poly-HRP80 Conjugate diluted to 50ug/ml in Stabilizer 85R-112 (#50-125-3383). Thermo Scientific Pierce TMB Substrate Kit (#PI34021). Normal recombinant cow Prion protein, PrP, was purchased from Abcam (#ab753).

III.b Antibody-coated Magnetic Beads

The magnetic beads were prepared using the Pierce NHS-activated magnetic beads IgG immobilization kit. To start, the protein solution and magnetic beads were brought to room temperature. 300 µL of beads were put into a microcentrifuge tube and put onto a magnetic stand to remove the supernatant. Then, 1 mL of ice-cold Wash Buffer A was added and mixed, after allowing the beads to congregate and the supernatant removed. Then, 200 µL of 8H4 Anti-PrP Antibody (Millipore Sigma, # P0110-200UL) solution was added and vortexted with an incubation of 1-2 hours at room temperature on a rotator. During the first 30 minutes, the tube was vortexed for 15 seconds every five minutes. For the remaining incubation, the tube was vortexed for 15 seconds everything 15 minutes. The beads were washed with 1 mL of Wash Buffer B added, mixed, and then placed on the rack to discard the supernatant. This was done a total of two times. 1 mL of ultra-pure water was then added to the beads, mixed, then placed on the rack and the liquid discarded. 1 mL of Quenching buffer was then added, mixed for 30 seconds, and incubated for two hours at room temperature on the rotator. Once done, the tube was placed in the magnetic rack and the beads allowed to aggregate and the liquid removed. Then it was washed with 1 mL of ultrapure water once. After this, 1 mL of storage buffer was added and mixed, then the liquid discarded, repeating this was twice. Finally, the beads were stored in the 300 µL of the storage buffer at 4°C until ready for use.

III.c Aptamer design and modifications

Aptamer DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, Iowa). Two LNA-modified version were designed: 3'-end biotin conjugated (5'-CTT A+TG +TCC AGA ATG CCA AAC GAG ACG GGG GGG +AC+A ATA G/3'Bio/), or a 20 Poly-adenine 3' end for AuNP functionalization (AAA AAA AAA AAA AAA AAA AAC TTA +TG+T CCA GAA TGC CAA ACG AGA CGG GGG GG+A C+AA TAG). Upon receipt,

DNA oligonucleotides were reconstituted to 100 μM in 1X TE buffer. The solution was then aliquoted and stored at -20°C until use.

III.d Gold Nanoparticle preparation

The aptamer was brought to room temperature and then incubated on a dry heat bath at 95 °C for 10 minutes. It was then removed and allowed to refold at room temperature. The PEG-biotin-SH was reduced by adding 250 μL of TCEP reducing gel slurry into a microcentrifuge tube and centrifuging for one minute at 1,000 x g at 4°C, discarding the supernatant. It was washed with 250 μL of TE buffer and spun down three times. Then, 50 μL of Biotin-PEG-SH was added and the solution was incubated for an hour on a rotating shaker. After, it was spun down again for one minute and then functionalized with AuNPs. This was done by adding 300 μL of citrate stabilized AuNP to a microcentrifuge tube. Then 7.5 μL of reduced Biotin-PEG-SH, and 6 μL of poly-A aptamer were also added. This solution was then incubated at -20°C for 1.5 hours.

Functionalized nanoparticles were isolated by centrifuging at 12,000 x g for 30 minutes at room temperature, discarding the supernatant. Then, 300 μL of 0.1 M NaCl and 10 mM phosphate buffer was added to disperse functionalized AuNPs. This was then spun for 30 minutes again and repeated for a total of three times. The beads were then stored in 300 μL of 0.3 M NaCl in 10 mM sodium phosphate buffer at 4 °C and protected from light.

III.e Enzyme Linked Oligonucleotide Assay

First, the 8H4-functionalized magnetic beads were blocked with 400 μL of Blocking Buffer and incubated for 60 minutes on a shaking incubator. The buffer was then removed and the specified bovine PrP was added and incubated for another 60 minutes on the shaking incubator. The beads were then allowed to aggregate and supernatant was discarded. The beads were then washed twice and transferred to a round bottom 96-well plate where they were washed a final time. After, 100 μL of 1 μM LNA aptamer or 1:5 AuNP solutions were added, then allowed to incubate for 60 minutes on an orbital shaker at room temperature. This was then removed and the beads were washed with wash buffer three times. Next, 100 μL of 0.2 μg/mL Steptavidin Poly-HRP80 was added and incubated for 60 minutes on the orbital shaker. This was then removed and washed three times, then transferred to new wells and washed again. Once the wash buffer was removed, 50 μL of wash buffer was added to disperse the beads and then 100 μL of TMB to each well. At desired end point, OD_{655} was measured using a plate reader.

IV. Results

The first experiment ran determined the sensitivity of the aptamer to PrP based on its configuration. The 17OAP1-24 aptamer was compared to an LNA version (**Figure 1**). While the negatives remained similar, there was a significant increase in signal with the LNA aptamer in comparison to the unlocked version in the 10 ng/ml PrP condition. It should be noted that the negative conditions were not conducted in duplicate, so there are no error bars. Therefore, these results suggest that the LNA version of the aptamer has a higher sensitivity to PrP in comparison to the unlocked version.

Upon finding the ideal aptamer modification, gold nanoparticles functionalized with the aptamer and biotin was tested. As shown in **Figure 2**, the functionalized gold nanoparticle had increased signal in comparison to the biotinylated aptamer. Additionally, ratios of aptamer and biotin were tested to determine if there was an optimal proportion for binding and signal. The goal was to achieve a surface area that contained the most efficient amount of biotin molecules to bind streptavidin. **Figure 2** shows that overall, the different ratios tested do not appear change the sensitivity of the immunoassay. As noted in the figure description, the ratio 1:5 of aptamer to biotin tested was not included in the graph, as they performed similarly.

The next experiment carried out was to determine the sensitivity of the assay to PrP with the modifications made from previous results. **Figure 3** indicates that the concentration used in past experiments of 50 ng/mL is not needed, and lower concentrations can be used. Specifically, the data shows that the 5 ng/mL concentration of PrP is sufficient to detect a significant difference between the control group and the experimental group. Notably, there is at least 5 standard deviations between the control group and the 5 ng/mL group, indicating that even lower concentration of PrP would likely suffice as well. Overall, this indicates that the changes to the immunoassay increased its sensitivity to PrP. The graph also shows that there is a nearly a 2.5-fold difference between the 5 ng/mL and 50 ng/mL condition. Though a 5-fold difference is theoretically expected, this is still a promising result.

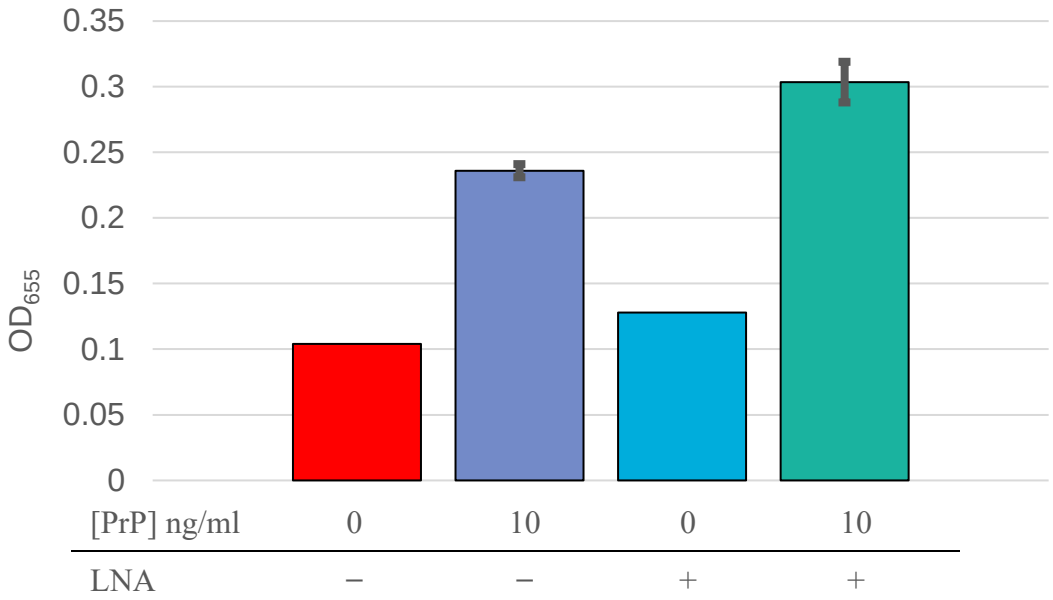

Figure 1- Optical density readings comparing unmodified and LNA-modified aptamer after PrP treatment. PrP containing samples were tested in duplicate (n = 2). Error bars signify standard deviation. OD_{655}: Optical density at 655 nm.

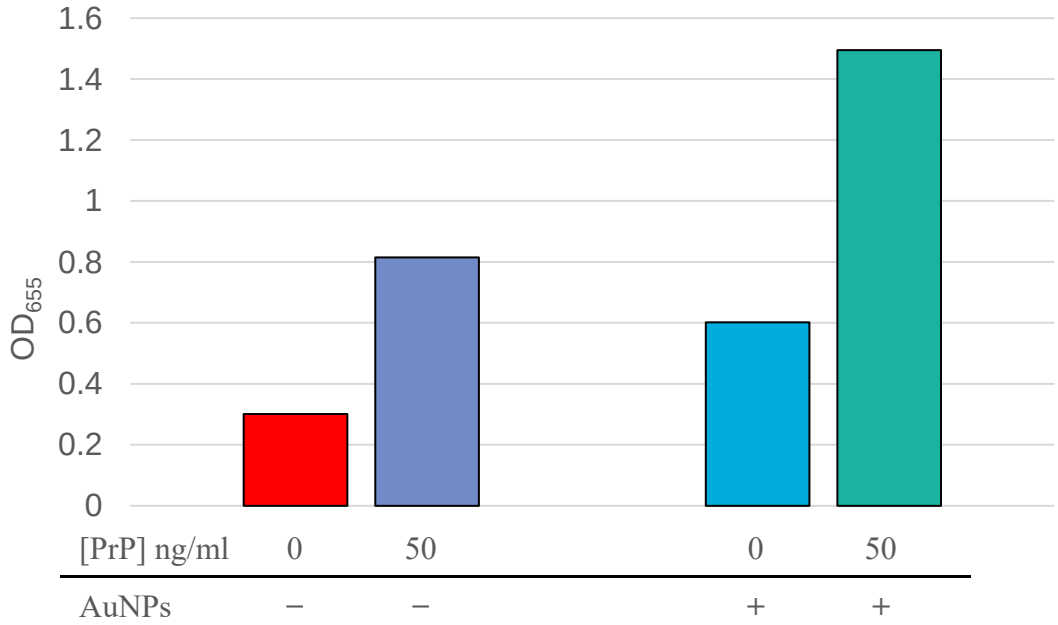

Figure 2- Optical density readings comparing LNA-modified aptamer to gold nanoparticles functionalized with aptamer and biotin. Four minutes post-TMB comparing aptamer/biotin functionalized AuNPs. Gold nanoparticles functionalized with aptamers containing varying concentrations of 20A-long 3' ends were tested, ratios 1:1, included on the graph and 1:5, not included on the graph as the results were very similar.

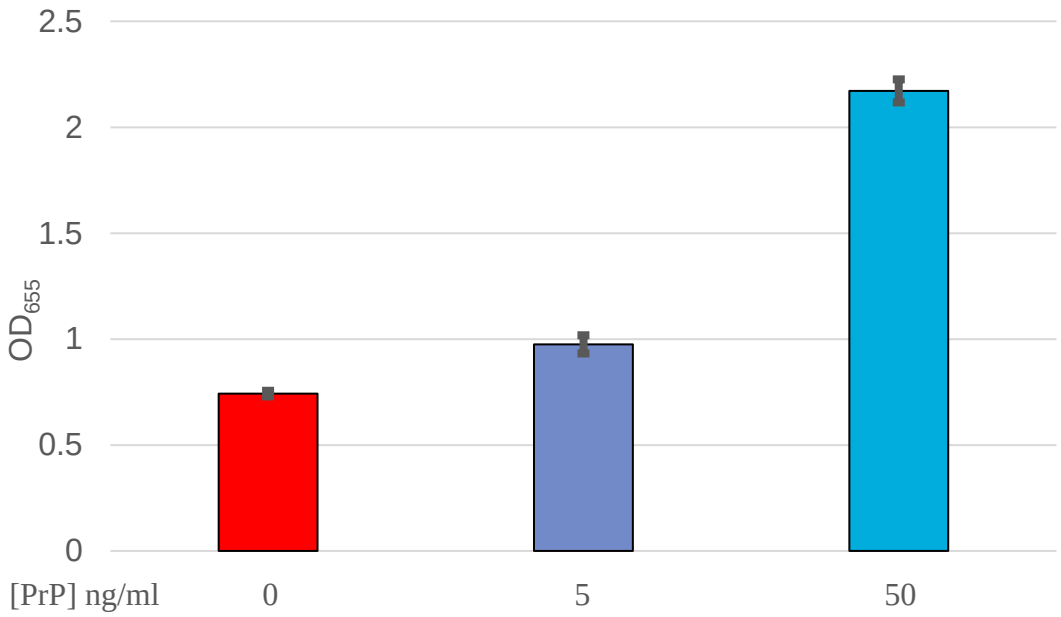

Figure 3 – Comparison of PrP concentrations. Gold nanoparticles functionalized with aptamers containing 20A-long 3' ends and thiolated biotin. Samples were run in duplicate, with the average being portrayed in the bar graphs. The standard deviation is shown though the error bars.

V. Discussion

To successfully increase the sensitivity of the ELONA, LNA aptamers and gold nanoparticles are used to increase stability and selective binding of the system. With respect to the current study, the goal was to improve the sensitivity of an immunoassay to detect prions. The LNA version of 17OAp1-24 was investigated (**Figure 1**) to determine if the stable structure affected the specificity of the aptamer. This yielded increased signal production from the LNA version by three standard deviations. Therefore, it can be concluded that the locked formation is important in increasing binding. Using LNA aptamers to increase signal has also been observed in other studies such as Jogensen et. al (2014). They used an aptamer with a LNA modification in the 3' end stem of streptavidin-binding site and observed a two-fold increase in binding affinity.

Functionalization of aptamers and biotin on gold nanoparticles were shown to increase the sensitivity of the immunoassay. Recent studies on COVID-19 also showed this phenomenon, using gold nanoparticles functionalized with aptamers to detect the spike protein in samples as low as 16 nM (Aithal et. al, 2022). Further investigation of biotin-aptamer ratios provided limited information regarding the most efficient ratios (**Figure 2**). While little information was gained from the two ratios tested, further exploration of this idea is suggested as altering the amount of biotin and aptamer would theoretically affect the amount of interaction with streptavidin. Testing other ratios could determine if alternative dilutions would present different results, as it is possible that the surface is saturated with aptamer/biotin in the two ratios tested in this study.

Detection of PrP down to 5 ng/mL was also observed (**Figure 3**). This is important, as postmortem clinical samples typically contain lower levels of PrP in comparison to postmortem tissue, such as the brain. Therefore, research pertaining to concentration of CWD PrP in antemortem samples is typically compared to the brain concentrations postmortem, present in concentrations that are around 10^-7 lower (Henderson et al., 2015). Specific amounts are largely unknown, but based off of the literature, it can be inferred that antemortem samples will have significantly less concentrations, so detecting lower amounts with this immunoassay is important. The logistics of these results are also important, as they imply that the amount of PrP and gold nanoparticles used can potentially be lowered, which pertains to the logistics of the development and materials used for the test.

When interpreting these results, it should be taken into account that the samples used were neat lab samples, meaning they were not environmental samples such as blood, homogenized tissue, or soil. Samples that are not as clean could affect the sensitivity and detection of the proposed testing assay. Additionally, this immunoassay was developed specially against one target, but there are many others that could be explored and used, such as bacterial particles (Wadhwa, et al., 2012; Hatate, et al., 2021).

These findings can be applied to other scientific topics and research, such as CWD testing and other pathogenic particles. As the experiments performed were carried out with a nonpathogenic version of this protein, these results can be used as a basis for the development of

an antemortem (live animal) test. In addition, the ideas presented in this paper to increase sensitivity can be applied to more than the ELONA. Other common immunoassays, such as IHC and ELISA, would likely benefit from combining the test and these findings. It is also possible that these findings could be applied to the detection of other pathogens.

In conclusion, this study has shown that modification of the 17OAp1-24 aptamer with LNA and gold nanoparticle has led to increased sensitivity and targeting within the ELONA. It is anticipated that future optimization with varying changes to these factors will further increase sensitivity.

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