Fluorescent and Scanometric Ultrasensitive Detection Technologies with the Bio-Bar Code Assay for Alzheimer's Disease Diagnosis

Undergraduate Researcher
Sadiya Khan, Northwestern University

Faculty Mentors
William Klein
Department of Neurobiology and Physiology, Northwestern University

Chad Mirkin
Department of Chemistry, Northwestern University

Postdoctoral Mentors
Lei Chang
Department of Chemistry, Northwestern University

Dimitra Georganopoulou
Department of Chemistry, Northwestern University

Abstract

Alzheimer's disease (AD) is a progressive mental disorder that affects 23 million people worldwide. It is pathologically characterized by amyloid plaques and tau tangles that form in the brain. The hard, insoluble amyloid plaques develop from the naturally secreted amyloid beta peptide, which can also assemble into toxic oligomeric forms known as amyloid beta derived diffusible ligands (ADDL). Currently, with no cure or a 100 percent accurate diagnostic method for AD, the potential for preventive medicine is limited. The presence of soluble ADDL in biological fluids of Alzheimer's patients, however, provides a promising biomarker for the use in detection. The application of functionalized nanoparticle technology in the use of protein detection, in this case ADDL, can be used for Alzheimer's disease detection. The ultrasensitive nature of this technique developed by the Mirkin lab, termed the bio-bar code assay, provides new hope for diagnosis. Using nanoparticles and magnetic microparticles functionalized with ADDL antibodies, this assay will allow for early diagnosis before irreversible brain damage has occurred. The sensitivity of the assay relies on an amplification step that results in thousands of DNA strands for each target protein. In conjunction with the bio-bar code assay, fluorescent and scanometric detection methodologies were used to detect the neurotoxic synthetic ADDL. Fluorescent detection was found to be highly quantitative but was not as sensitive as the scanometric method. The possibility of improving the sensitivity of fluorescent detection can be further explored using a fluorophore Alexa-488 tagged bar-code DNA. The sensitivity of the scanometric method proved useful in showing a difference in plasma ADDL levels between Alzheimer's patients and control samples, providing a promising beginning for the early detection and diagnosis of this debilitating neurodegenerative disease.

Introduction

Currently more than 23 million people worldwide suffer from the neurodegenerative condition of Alzheimer's disease (AD).[1] Approximately 54,000 deaths were attributed to this fatal disease in the United States alone in 2001.[2] Dementia is the leading clinical symptom. The average life expectancy following diagnosis is estimated at eight years.[3] Age is a leading risk factor, with AD attacking one in two Americans over the age of 85. It is projected that 14 million Americans will be affected by 2050, with financial costs for Alzheimer's patient care inevitably rising from the present annual $100 billion.[4] AD is currently the third most expensive disease in the United States, following heart disease and cancer. These startling numbers are evidence of an enormous public health problem and a pressing need to pave the way for future treatment options by gaining a better understanding of how this disease affects the brain.

The human brain is composed of billions of neurons that conduct millions of messages a minute. Each neuron boasts an approximately 100-year life span, making the death and regeneration of neurons unlike other cell types. As neurons are destroyed in AD, neurogenesis is not able to replace all the lost cells. During the early stages of AD, the progressive loss of neurons in the hippocampus affects short-term memory and the ability to perform routine tasks. As the disease spreads to the cerebral cortex, language skills decline and behavioral changes are noticed. In AD's final stages, patients completely lose the ability to communicate and recognize friends and family members and are completely dependent on the care of others.

The pathology of AD is characterized by intraneuronal tangles and extracellular plaques in the brain. Neurofibrillary tangles are composed of altered forms of a protein called tau. Tau is normally found inside brain cells as part of a structure called a microtubule that is responsible for transporting nutrients. In Alzheimer's the tau protein is either phosphorylated or truncated, which leads to the formation of tangles and the destruction of neurons. The neuritic plaques, on the other hand, develop in the brain between nerve cells as a result of the accumulation of amyloid beta peptide. The hard, insoluble deposit that forms as a result of this protein accumulation interrupts synaptic communications between neurons. Additionally, acetylcholine, a neurotransmitter responsible for processing memory and learning, is present in depleted levels in AD brains, further preventing synaptic communications.

Fluorescent and Scanometric Ultrasensitive Detection Technologies with the Bio-Bar Code Assay for Alzheimer's Disease Diagnosis *(continued)*

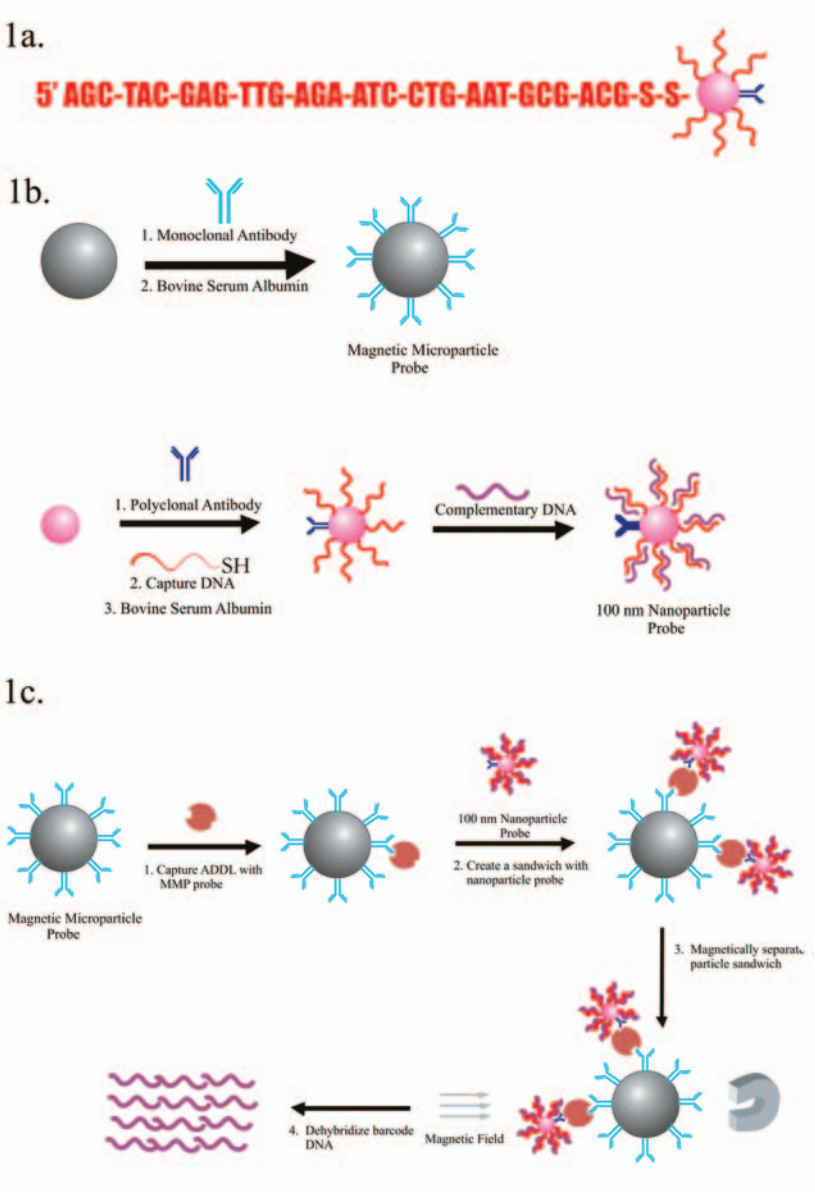

Figure 1: The schematic for the bio-bar code assay shown here includes synthesis of probes and protein detection.

The only way to accurately and confidently diagnose Alzheimer's at present is through a brain autopsy to evaluate the presence and frequency of tau tangles and amyloid plaques. As this method of detection clearly cannot assist in patients seeking treatment options, alternatives need to be found. Clinical diagnosis currently relies on neurophysiological tests along with positron emission technology (PET) and magnetic resonance imaging (MRI) scans. However, scans can be costly and time inefficient, and they yield an AD diagnosis specificity of only 63 percent.[5] Since AD is responsible for more than 50 percent of all dementia cases (the remaining causes include treatable and reversible causes such as vitamin B_{12} deficiency), the positive identification of AD is crucial to providing proper medication and treatment options to patients. Though there is no cure at present, drugs that have been shown to slow the progression of Alzheimer's are available. Therefore, early detection and diagnosis are critical so that patients and their families can make informed choices about their options before irreversible nervous damage.

The bio-bar code assay provides an ultrasensitive technique to detect the soluble ADDL.[6] This small neurotoxic molecule forms fibrils, the precursors of the amyloid plaques, and is believed to be responsible for memory loss in AD. The solubility of ADDL suggests the presence of this oligomer in bodily fluids such as cerebrospinal fluid and possibly blood. This makes ADDL an ideal biomarker for the development of a reliable and noninvasive diagnostic tool. Additionally, earlier detection provides the opportunity for further research on the changes in the brain during AD.

Background

While symptoms and indicators are known, the cause of Alzheimer's disease is still greatly debated. The amyloid cascade hypothesis proposed 10 years ago states that the accumulation of amyloid beta peptide results in the amyloid plaque observed in AD patients. The remaining components of the disease such as tau tangles occur then as a result of the formation of the plaques. The weakness of the old amyloid hypothesis is that there is no consistent correlation between the number of amyloid deposits and cognitive impairments, as some humans show many cortical amyloid deposits without AD.[7]

Conversely, the second hypothesis implies the opposite — that neurofibrillary tangles form first, inducing the formation of neuritic plaques. However, in studies with mutated tau genes, the formation of severe neurofibrillary tangles that led to the degeneration of neurons failed to invoke deposits of amyloid plaques.[8] On the other hand, research conducted with mutated amyloid protein genes indicated that the formation of tau tangles follows the amyloid plaque formation.[9] This was confirmed by another study that showed that the amyloid beta peptide promotes the formation of tangles by triggering the cleavage of tau.[10]

Recently the amyloid cascade hypothesis was modified to also include neurotoxic amyloid beta assemblies composed of soluble amyloid Aβ oligomers. This molecule, termed ADDL, was shown to have a startling ability to attach to and destroy neurons. While the monomer of the amyloid peptide was found to be innocuous, the self-assembly of fibrils proved to be highly neurotoxic. These fibrils are precursors of the insoluble plaques and cause memory loss. Thus, neurological damage can be directly related to the presence of ADDL. While there was little correlation between amyloid plaque deposits and cognitive functioning, the levels of ADDL were found to reach up to 70 times more in AD brains than in control brains.[5]

The remarkable reversal of memory failure in a transgenic mice model using an Aβ-directed antibody points to the possibility of a cure through a simple vaccination.[11] Although there is no evidence that this method can improve the cognitive functioning of Alzheimer's patients, the immunoneutralization of the soluble amyloid beta oligomers resulting in the reversal of memory loss confirms ADDL's neurotoxicity and supports its role as the trigger for AD. This indicates that the molecule ADDL could be a powerful biomarker for the early detection of Alzheimer's, allowing more AD patients access to developing treatments.

The field of nanotechnology shows great promise in the biodetection of protein analytes. A localized surface plasmon resonance biosensor using optical properties of gold nanotriangles showed a sensitivity of ADDL detection to 100 pM.[12] In comparison with the Enzyme-Linked ImmunoSorbent Assay (ELISA), this assay is up to a million times more sensitive.

The bio-bar code assay developed by the Mirkin group can detect protein concentrations down to the attomolar range.[13]

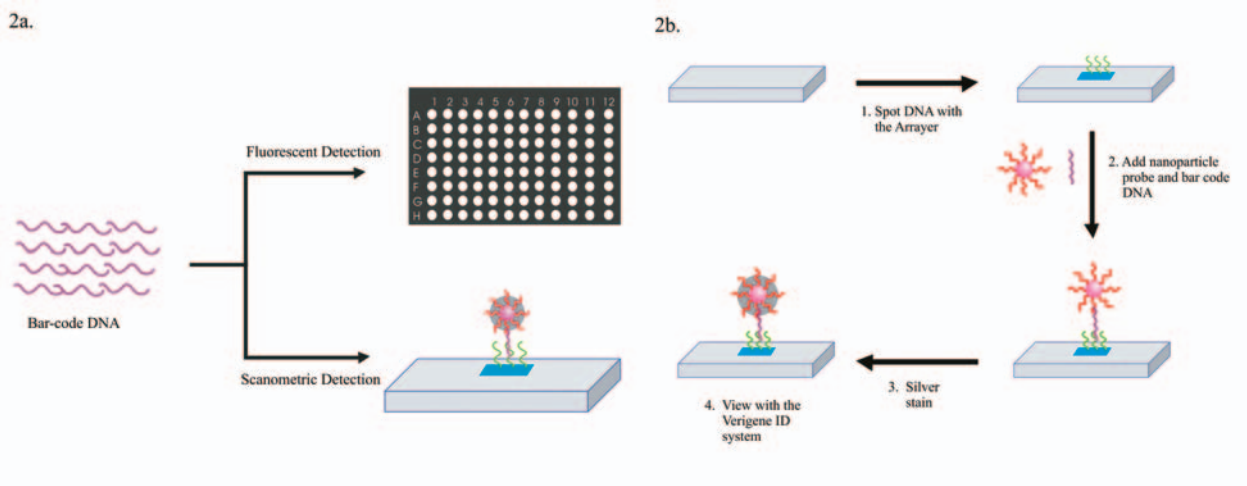

Figure 2: The two methods of DNA detection used are shown here with the scanometric schematic as developed by the Mirkin lab.

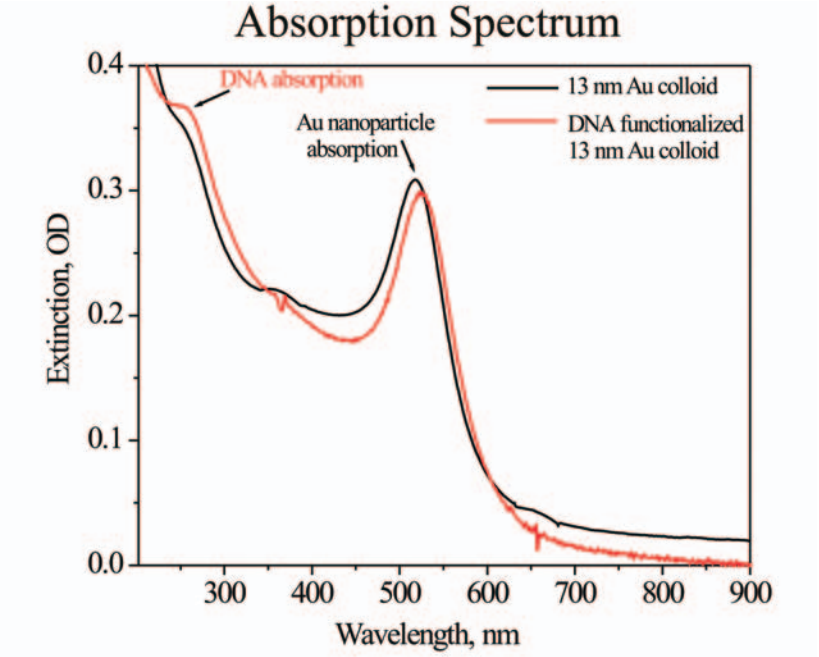

Figure 3: Absorption spectrum of 13 nm gold colloid compared with the absorption spectrum of DNA functionalized 13 nm gold colloid.

Used for a variety of diseases from mad cow disease to HIV, the amplification of the signal by the detection of DNA, rather than the detection of the protein, allows for accurate and sensitive results. With increased sensitivity, there is increased promise for early detection of the ADDL molecule in biological fluids.

Approach

Synthesis of Probes

The bio-bar code assay requires antibodies, magnetic microparticles, nanoparticles, and DNA. The ADDL-specific monoclonal (20C2) and polyclonal (M90) antibodies were generated in rabbits.[14] Amine-functionalized magnetic microparticles were obtained from Polysciences, Inc. Gold nanoparticles of diameter 13 nm were synthesized by citrate reduction of gold salt ($HAuCl_4$) in house and characterized by ultraviolet and visible spectroscopy (UV/Vis) as well as transmission electron microscopy (TEM). Bigger nanoparticles (100 nm) were purchased from Ted Pella. DNA oligonucleotides were prepared on an automated synthesizer (Expedite), purified by high-pressure liquid chromatography (HPLC, Hewlett Packard), and characterized by UV/Vis (Asco Instruments). DNA oligoncleotides were also purchased from Integrated DNA Technologies (IDT).

Following the synthesis of the individual components, the next step required the synthesis of the ADDL-specific probes needed in the assay. Magnetic microparticles were first activated and then functionalized with monoclonal anti-ADDL in 0.1M NaCl PBS with 0.1 percent bovine serum albumin (BSA). The 100 nm particles were functionalized with thiolated DNA (Figure 1a) and polyclonal anti-ADDL using a slow salt-aging method for 48 hours, reaching a final salt concentration of 0.1M NaCl and 0.01M phosphate buffer pH 7. Complementary bar-code DNA was then added and hybridized to the already present DNA (Figure 1b). The complementary DNA added was modified with dyes, FAM or Alexa-488, for use in fluorescent detection. The use of gold 100 nm particles in this assay eliminated the need for a polymerase chain reaction (PCR) step while maintaining sensitivity due to the large ratio of bar-code DNA released for one target molecule.

The ADDL molecules were synthesized in DMSO with Aβ 1-42 peptide (California Peptide Research, Napa) in the Klein lab. Following the probes and ADDL preparation, the bio-bar code assay was conducted with subsequent detection of the DNA that was released from the assay.

The Bio-Bar Code Assay

The bio-bar code assay consists of two binding events. The first event is the recognition and attachment of ADDL to the monoclonal anti-ADDL on the magnetic microparticle. This complex is magnetically separated from any unbound molecules and repeatedly washed in 0.1M NaCl phosphate buffered saline (PBS) with 0.1 percent BSA. The second binding event is the recognition and attachment of the polyclonal anti-ADDL on the gold nanoparticle to the protein that is already attached to the magnetic microparticle. This sandwich of ADDL,

nanoparticle, and magnetic microparticle is again isolated through magnetic separation and washed repeatedly. Finally, with the addition of water, the complementary DNA on the nanoparticle dehybridizes and is released (Figure 1c). With increasing ADDL concentration, greater numbers of ADDL-magnetic microparticle-nanoparticle complexes are created. The more complexes created, the more DNA released. The ADDL signal is thus amplified through the thousands of DNA strands released for each ADDL molecule.

DNA Detection

To complete the detection of ADDL following the bio-bar code assay, the detection of the DNA released is conducted. Two different methods for detection were explored: fluorescent and scanometric (Figure 2a). Fluorescence is a property of some molecules to absorb light at a particular wavelength and emit at a longer wavelength. To quantify the concentration of the fluorescence present, the area under the emission curve is used. There exists a proportional relationship between emission area and the amount of fluorescence. The fluorescent detection involved hybridizing fluorescently tagged DNA to the nanoparticle. The fluorescent tags that were used included FAM and Alexa-488. Following the assay, the fluorescent DNA released was quantified at the Keck Biophysics Facility using a Gemini EM Fluorescence/Chemiluminescence Plate reader. This method is quantitative and time efficient (less than five minutes).

The scanometric method requires a glass slide and an additional gold 13 nm probe. An oligonucleotide sequence that is half complementary to the bar-code DNA collected in the assay is spotted with a microarrayer on the glass slide. An arbitrary noncomplementary oligonucleotide sequence is also spotted to provide a negative control on the slide. The 13 nm gold probe is functionalized with an oligonucleotide sequence that is also half complementary to the DNA released by the assay. The bar-code DNA from the assay thus hybridizes both to the glass slide and the gold nanoparticle to form a new sandwich. Silver staining the slide following a Ted Pella protocol results in silver deposition only at the places where gold nanoparticles are present due to the catalytic activity of gold (Figure 2b). The more bar-code DNA is present, the more gold particles are deposited, resulting in an increase of intensity of the spots. It should be emphasized that this method is highly sensitive to the attamolar range.

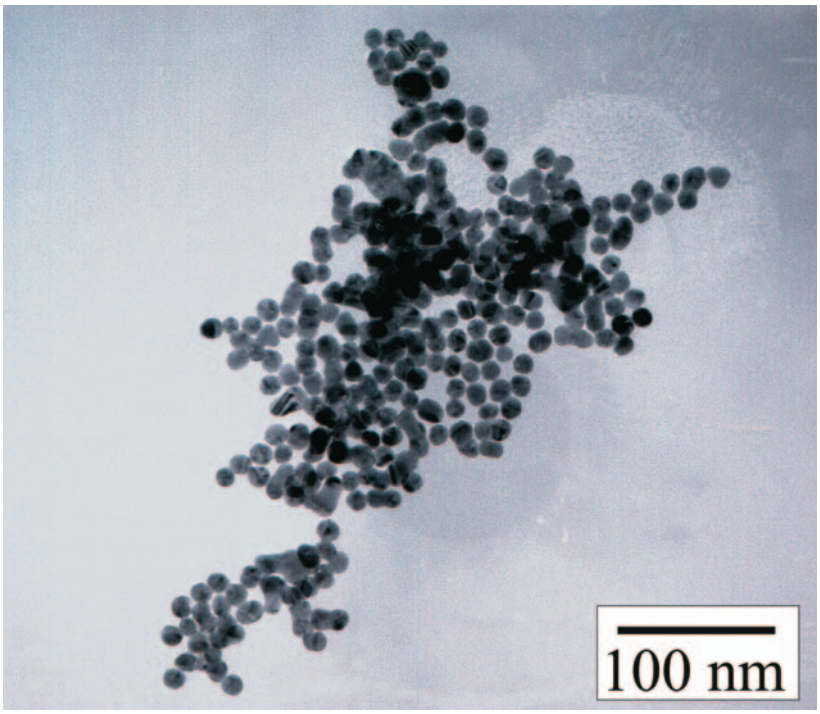

Figure 4: A TEM image of 13 nm gold particles emphasizes size and spherical shape.

Patient Sample Evaluation

After the scanometric method was established as a more sensitive DNA detection method, patient plasma samples were evaluated for a difference in signal intensity between control and AD patients. Plasma samples were obtained from Northwestern University Alzheimer's Brain Bank and stored at -80° C. The bio-bar code assay was conducted on the samples followed by scanometric detection.

Results and Discussion

Gold nanoparticle synthesis was evaluated by UV/Vis, which shows a peak at 518 nm characteristic for 13 nm gold particles.

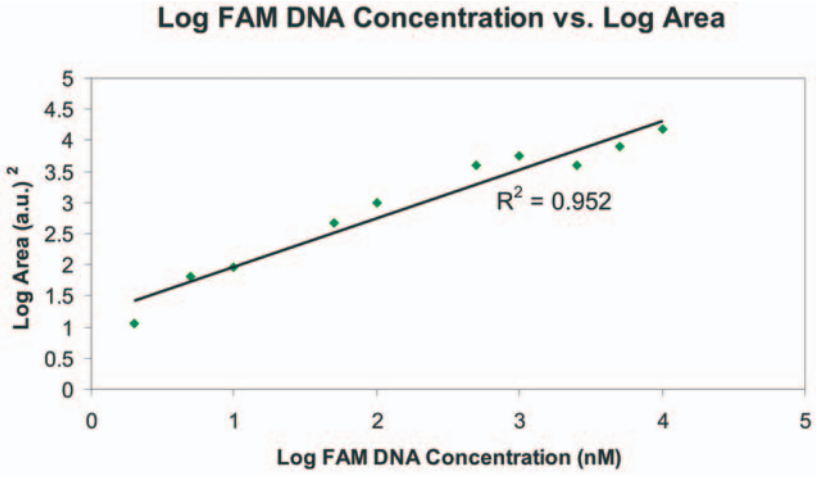

Figure 5: Standard curve for FAM-labeled DNA exhibiting the linear relationship between fluorescent DNA concentration and emission area.

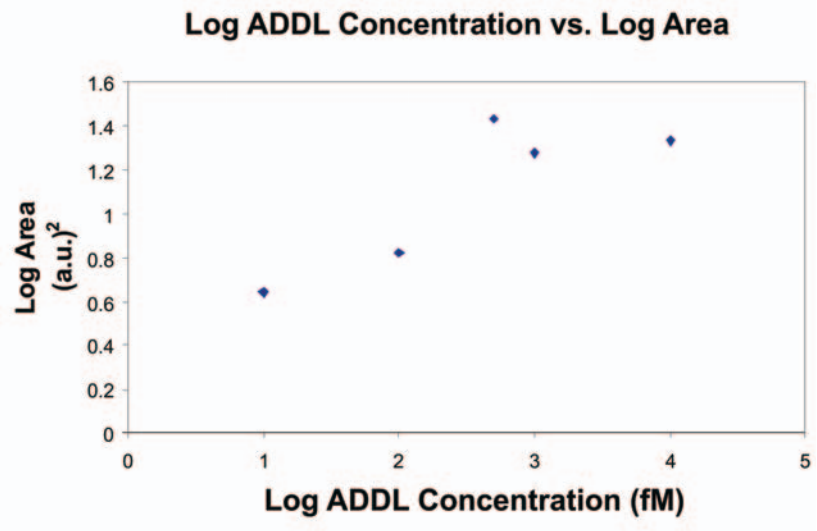

Figure 6: Increasing fluorescent trend for the FAM-labeled bar-code DNA collected from the bio-bar code assay for ADDL detection.

Following DNA functionalization, an additional peak is observed at 260 nm corresponding to the DNA (Figure 3). The TEM image presented in Figure 4 indicates the spherical shape and the size of the particles. The gold nanometer probe size used in the bio-bar code assay was 100 nm, since the number of DNA strands is on the order of magnitude of thousands per particle, as opposed to approximately 100 strands on the 13 nm particle. With a larger surface area on the bigger particle, more strands of DNA can attach, further amplifying the signal of each target molecule.

Endpoint fluorescence was first conducted for a serial dilution of fluorescently tagged DNA, resulting in a linear range in high nanomolar concentrations. However, at lower concentrations overlap from the absorbance peak limited the sensitivity of endpoint fluorescence. The area under the emission spectrum, however, is proportional to the concentration of the fluorescence over a larger range, providing a quantitative method for determining concentration. The first step was to optimize a standard curve by measuring the emission area for a known set of concentrations of FAM-tagged DNA (Figure 5). Solutions were always mixed for five seconds prior to reading. After several standard runs, it was concluded that the ideal conditions for fluorescence detection of DNA concentrations in the range 100nM to 0.5nM required a minimal volume of 100uL in each well.

Once a linear standard curve had been obtained for the FAM DNA dilutions, the bio-bar code assay was conducted with FAM-labeled nanoparticles. Synthetic ADDL was prepared on a weekly basis, and a set of known dilutions were evaluated with the assay. As seen in

Figure 6, an increasing fluorescent trend was observed, with increasing ADDL concentration in the range from 10 fM to 10 pM.

Problems reproducing the results from the assay came from two main problems with the fluorophore FAM. FAM is highly susceptible to photobleaching and pH. The optimal pH for FAM is 9, and its fluorescent intensity decreases at pH 7, which is the physiological pH at which the bio-bar code assay was conducted. This resulted in inaccurate and nonconsistent results. To resolve these problems, a new fluorophore, Alexa-488, was explored. Alexa is highly insensitive to photobleaching, with an extinction coefficient of 41,000 and pH stability within the 4–10 range. This results in increased intensity, allowing increased sensitivity in the detection of Alexa-labeled DNA and ultimately ADDL.

Preliminary data with a standard set of Alexa-labeled DNA dilutions in the picomolar to nanomolar range show a linear relationship between emission area and concentration of the fluorophore-tagged DNA (Figure 7). In addition to the standard curve, the bio-bar code assay was conducted with nanoparticle probes functionalized with Alexa-labeled DNA to detect synthetic ADDL. A linear trend in increasing fluorescence was observed from 10 attomolar to 10 picomolar as seen in Figure 8, showing promise for a highly quantitative and highly sensitive method of ADDL detection. The reproducibility and consistency of these results have to be further examined.

The second method of DNA detection that was explored for ADDL detection was scanometric. This detection method developed by the Mirkin group provides extremely high levels of sensitivity but is

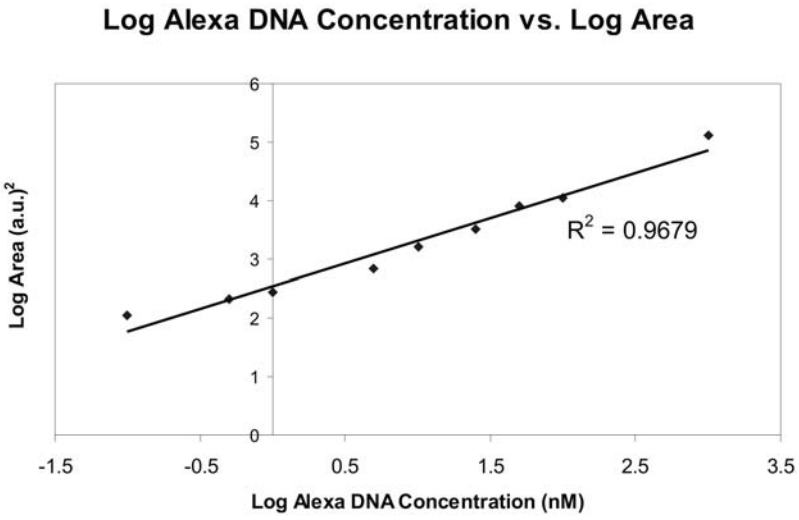

Figure 7: Preliminary results for standard Alexa-labeled DNA curve showing a linear relationship between Alexa-labeled DNA and emission area from picomolar to nanomolar range.

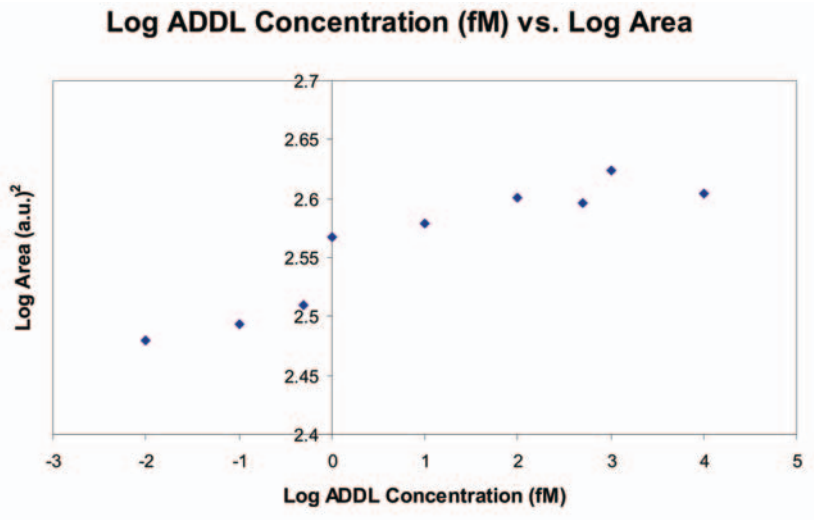

Figure 8: Preliminary data from Alexa-labeled bar-code DNA from the bio-bar code assay for ADDL.

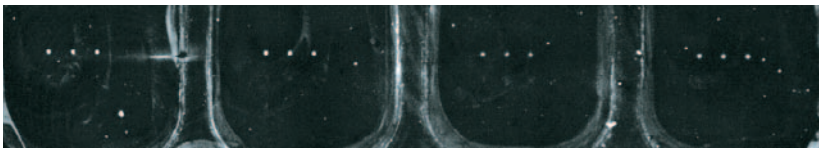

Figure 9: Photograph taken by the Verigene ID System of spotted glass slide following bio-bar code assay and scanometric and silver staining with the white spots, indicating the presence of bar-code DNA.

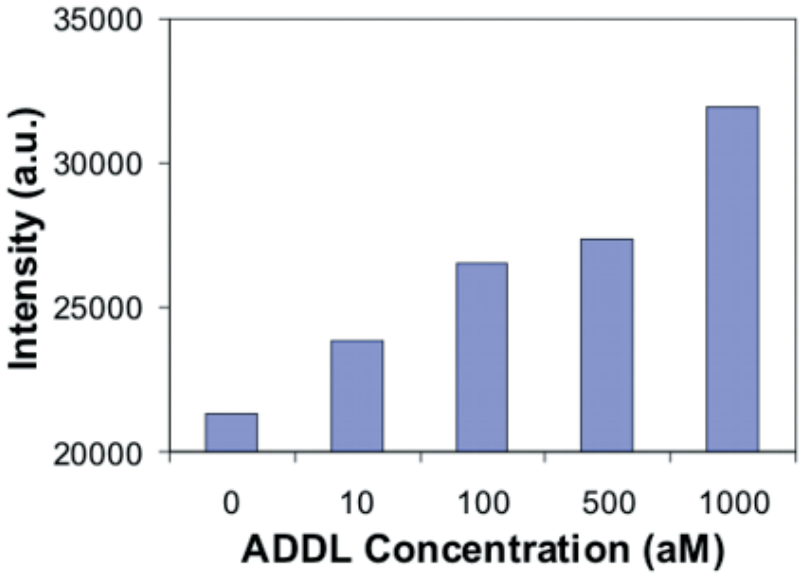

Figure 10: Increasing trend of spot intensity with increasing ADDL concentrations shown here indicates the ultrasensitivity of the scanometric method. The assay has a dynamic range between 0–1000 aM.

susceptible to variable data due to increased background attributed to silver staining. The Ted Pella protocol for silver staining was used with an incubation time of about five minutes. This resulted in the detection of ADDL down to a 10 attomolar concentration. The photograph of the glass slide following silver staining was taken by the Verigene ID System (Figure 9). The presence of dots on the glass slide indicates the presence of the bar-code DNA that was isolated by the bio-bar code assay for ADDL.

Analysis of the intensity of the dots was conducted using microscopy analysis software. With increasing ADDL concentration, an upward trend of increasing intensity is observed in the dots (Figure 10). While absolute intensity values cannot be compared from slide to slide, the scanometric method provides a simple way of detecting the presence of ADDL at very low concentrations.

The scanometric method was next used to evaluate 40 patient plasma samples. Plasma is the ideal body fluid for clinical diagnostic tests because it is easy to obtain and relatively painless to collect. An overall difference in intensity was observed between the Alzheimer's patients and the control patients. These preliminary results indicated an elevated level of ADDL in the plasma of Alzheimer's patients and need to be repeated. Although we cannot quantify ADDL levels with this method at the moment, the ability to simply detect a difference is a major step towards an accurate and reproducible detection method.

Conclusions

Fluorescent and scanometric methods for DNA detection both proved successful in detecting ADDL at low concentrations. Fluorescence is a highly quantitative method and provides a quick way of detecting DNA. FAM-labeled DNA used in the bio-bar code assay was sensitive to ADDL in the range of 10 fM to 10 pM. However, results were hard to reproduce. Increased sensitivity and reproducibility of fluorescent detection can possibly be attained by further exploring a new fluorophore, Alexa-488. Scanometric detection was sensitive to a concentration of 10 aM of ADDL, despite the variability in intensity due to background. Plasma samples from control and Alzheimer's patients evaluated with the scanometric method showed that overall the Alzheimer's group had a greater intensity, indicating elevated levels of ADDL in the plasma. The use of ADDL as a biomarker with plasma is a hopeful beginning to a clinical diagnostic tool for Alzheimer's disease.

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