Utilizing the Scanometric Immunoassay for the Ultrasensitive Detection of Aβ-42 Oligomers for the Early Detection of Alzheimer's Disease

Undergraduate Researcher
Anita A. Sapre
University of Pennsylvania, Philadelphia, PA

Faculty Mentor
William L. Klein
Department of Neurobiology and Physiology
Northwestern University

Postdoctoral Mentor
Mary P. Lambert
Department of Neurobiology and Physiology
Northwestern University

Abstract

Alzheimer's disease (AD) is one of the most common forms of dementia, with no current definitive premortem diagnosis. Amyloid beta-peptide derived diffusible ligands, known as ADDLs, have become a key link in the AD specificity for memory loss and are a potential biomarker for AD. In order to develop an early diagnostic tool, ADDLs are a target of new ultrasensitive protein assays, such as the scanometric immunoassay (SIA). To utilize the SIA for ADDL detection, different detection antibodies and oleocanthal treatments were examined to improve the sensitivity of the assay. The oleocanthal treatments reduced background in the lower concentration wells and had no apparent effect on the detection probes. Control transgenic mouse brain extracts, control human brain extracts, as well as high-performance liquid chromatography (HPLC) fractionated extracts were also studied as part of the transition toward examining biological samples. Using the SIA, ADDLs were detected in both HPLC-fractionated control and AD human brain extracts using NU-1 as the capture antibody. This is noteworthy because the dot-blot assays on these samples had no signals for any fraction. Furthermore, the SIA was able to detect a 4-fold difference between the control and AD nonfractionated samples, while the dot-blot assay had only faint detections when using NU-1. These results make the SIA promising for detecting ADDLs in biological samples.

Introduction

The onset of Alzheimer's disease (AD) is characterized by an inability to form new memories and progresses into the deterioration of cognitive function.[1] It is the most common form of dementia in persons over the age of 60, affecting over 25 million individuals worldwide; however, there is no definitive premortem diagnosis.[2] Thus, AD research has focused on understanding the pathological mechanism and progression of the disease in order to develop techniques for immunotherapy and early diagnosis. Scanometric immunoassay (SIA) is a new ultrasensitive protein assay that may prove effective in detecting an AD biomarker.

The development of plaques and tangles are two distinguishing features of AD, which can be identified postmortem. These plaques and tangles are derived from the amyloid beta peptide (Aβ-42) and hyperphosphorylated tau, respectively.[2] Aβ-42 has played a critical role in hypotheses on the mechanism and specificity of AD for memory. These hypotheses include the original amyloid-cascade hypothesis along with the more recent inclusion of ADDLs.[2,3] Understanding the binding and specificity of ADDLs, along with the role of other associated proteins, has strengthened the link between ADDLs and the memory specificity of AD.[4] Thus, ADDLs are a potential pathogenic biomarker for AD, and precisely measuring their concentration is of interest in the development of a conclusive diagnostic measure.

Conventional protein assays, such as the enzyme-linked immuno-sorbent assay (ELISA), are not sufficiently sensitive to determine the less-than-1 pM concentration of ADDLS in the cerebrospinal fluid (CSF).[5] To overcome this limitation, different variations of potential biomarkers have been measured, including total tau protein, phosphorylated tau protein, and Aβ-42 protein.[1,5,6] Since the measurement of these markers has led to inconclusive results, more sensitive assays have been developed utilizing a nanotechnology-based, two-antibody sandwich protocol.[5] One of these assays is the bio-barcode assay, which has proved effective for proteins, such as the prostate-specific antigen and ADDLs, and for nucleic acids.[7] However, the recent development of the SIA may prove more reliable and effective for measuring ADDL concentrations in CSF for the diagnosis of AD.

Background

Aβ-42 Oligomers

The Aβ peptide is physiologically prevalent in the monomer form; however, the fibril, protofibril, and oligomeric forms are neurotoxins.[4] The amyloid-cascade hypothesis linked the fibril form of Aβ-42 to the development of AD. However, inhibitors of fibrillogenesis did not block neurotoxicity; thus, fibrils could not be the only toxic form.[2] The specificity of AD to memory has been attributed to the disruption of synaptic plasticity and the effects on long-term potentiation (LTP) rather than to direct neuron death.[4] LTP is an increase in transmission across the synapses that is closely linked to memory mechanisms. A critical link to memory-loss specificity in AD has been the identification of ADDLs, the small globular oligomeric form of Aβ-42.[3] The specificity of ADDL binding to a particular subgroup of neurons has been widely studied in order to understand the associated receptors and proteins. There have been studies on clusterin, trypsin, Arc, Fyn, PSD-95, NMDA receptors, and, most recently, insulin receptors.[4] As a potential biomarker, ADDLs are the target protein in this study utilizing the SIA.

Conformation-Specific Antibodies

The development of conformation-specific antibodies that can target a specific form or forms of Aβ has been of great interest for use in this ultraprecise assay. With conformation-specific antibodies it is possible to distinguish Aβ oligomers from the physiologically relevant Aβ monomers.[9] NU-1, NU-4, and M71/2, for example, have proved effective in distinguishing between AD and control samples.[8] Furthermore, these antibodies have different conformational epitopes, as confirmed in competition-dot immunoblots.[8] The presence of different conformational epitopes facilitates the use of these antibodies in sandwich assays, such as the SIA, which utilize two antibodies: a capture antibody and a detection antibody. However, distinct epitopes are not necessary when measuring ADDLs because they may have multiple binding sites due to their oligomeric nature.[8]

Related Assays

The ELISA is a conventional sandwich assay and has a limit of detection of 1 pM. The sandwich-ELISA assay utilizes a secondary antibody as a detection mechanism. The secondary antibody is linked to an enzyme that will change color or fluoresce when the secondary antibody is stimulated.

Although conventional ELISAs have limits of detection in the pM range, they are not sufficiently sensitive for ADDL measurements in the CSF. To overcome this limitation the bio-barcode assay can be utilized; it has a lower limit of detection of ~100 aM and an upper limit of ~500 fM for ADDL measurements.[5] This assay utilizes a similar antibody-sandwich mechanism; however, it also has two distinct features. The capture antibody is functionalized to a magnetic particle, allowing magnetic fields to be used as a separation technique. The detection antibody is functionalized to a gold nanoparticle with barcode DNA sequences; these sequences can be dehybridized, creating an amplification and detection technique.

Previous Clinical Studies

While the bio-barcode assay aims to measure ADDL levels, previous clinical studies have been conducted correlating elevated tau-protein levels and Aβ-42 levels with cognitive diagnosis methods such as the mini-mental state examination.[1] It was noted by Clark et al. that elevated tau levels can also be attributed to other forms of dementia, including forms of prion disease.[1] Similarly, a clinical study was conducted by Maddalena et al. to determine the diagnostic capabilities of measuring the ratio between phosphorylated tau and Aβ-42 in CSF.
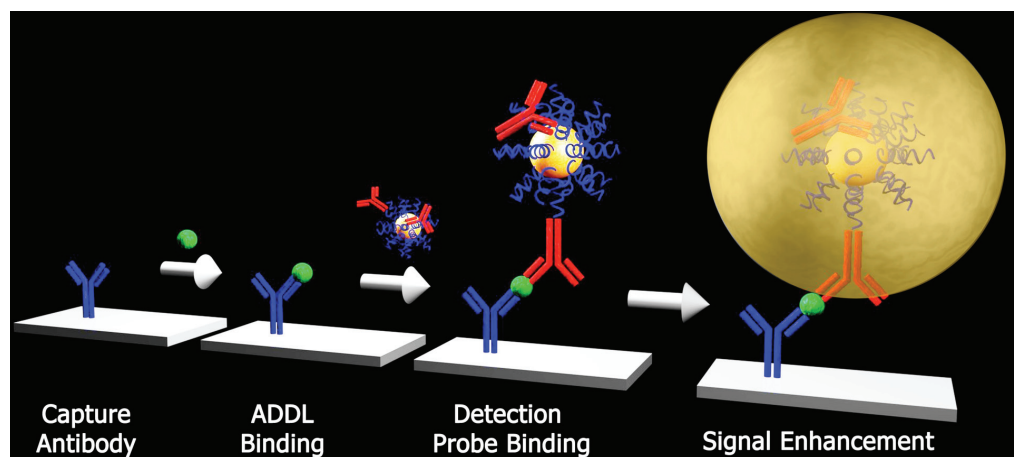
Figure 1. The four stages of the scanometric immunoassay. a) Capture antibody covalently immobilized on CodeLink Activated glass slide. b) ADDL target binds to capture antibody, 2-hour incubation. c) Detection probes bind to ADDLs, 2-hr incubation. The detection probe consists of a 13 nm Au NP functionalized with T24 oligonucleotide sequences. The oligonucleotide sequences facilitate the binding between the detection antibody and the Au NP. d) Slide development using reaction between $HAuCl_4$ and hydroxylamine to yield Au for nanoparticle enhancement. Detection using the Nanosphere Verigene Reader. (Image courtesy of Weston Daniel, Mirkin Group.)
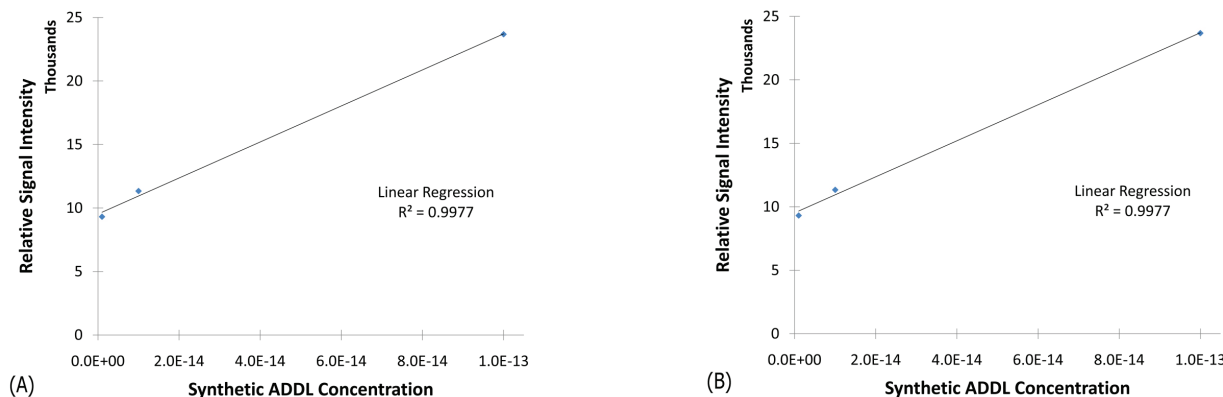
Figure 2. Standard curve with synthetic ADDLs using the M89/3 detection antibody and NU-1 capture antibody. 10-fold ADDL dilutions from 1 nM to 1 fM were used to develop the standard curve. Linear regressions of the 1 fM to 100 fM range and the 1 pM to 100 pM range yield R^2 factors of 0.9977. This also confirmed the functionality of the fresh T24 Au NPs and the M89/3 antibody.

This ratio was found to have higher diagnostic accuracy than either value alone; however, there was no correlation with the degree of severity of dementia.[6]

Since current diagnostic accuracy is less than 80%, and previous clinical studies on aggregate protein levels do not provide diagnostic accuracy, ultrasensitive detection assays are another diagnostic solution. Thus, the SIA can utilize ADDLs to develop a more definitive diagnostic tool for AD.[6,5]

Approach
The SIA follows a four-stage process outlined in Figure 1, and currently yields detection sensitivity for ADDLs of ~10 fM; thus, the process must be optimized to improve sensitivity. The SIA is similar to the bio-barcode assay and the sandwich-ELISA assay, since it utilizes a two-antibody sandwich process to detect the protein target. However, the SIA differs from both assays in its separation and amplification techniques.

As seen in Figure 1, the SIA sandwiched the ADDL targets between an immobilized capture antibody and a detection antibody probe complex. The capture antibody was covalently functionalized onto the activated glass surface using an arrayer. The detection probe complex consisted of a 13 nm gold (Au) nanoparticle (NP) functionalized with T24 oligonucelotide sequences. The oligonucleotide sequences were necessary to facilitate the binding of the detection antibody to the Au NP. After the two antibodies were bound to the ADDLs, creating the sandwich complex, the Au NPs were amplified with Au for visible detection. The Au amplification was the result of a reaction between hydroxylamine and $HAuCl_4$ in a 2:1 ratio. The Verigene Reader (Nanosphere) was used for detection of the signals, and image analysis software was used for quantitative comparison of the relative signal intensity. The amplification and detection process was completed twice for increased signal strength.

One of the major advantages of the SIA was the immobilization of the capture antibody on the CodeLink Activated glass slides. The spotting of the capture antibody allowed for precise deposition. Furthermore, the immobilization facilitated the many washing stages; since the sandwich complex was affixed to the slide, a magnetic separation technique was not necessary.

Results and Discussion

*Developing a Standard Curve with ADDL-Concentration-Dependent Response*
The initial experiments using the SIA were used to determine if a response curve would be dependent on ADDL concentration for the 100 pM to 1 fM range. The SIA was conducted using NU-1 capture antibody spotted on the slides and M89/3 detection antibody conjugated to the Au NP probes. Synthetic ADDLs were serially diluted in SIA buffer, in 10-fold increments. Figure 2 is a representative standard curve that was obtained. Linear regressions on the 1 fM to 100 fM range and 1 pM to 100 pM range had R^2 factors of 0.9977, indicating a strong correlation between concentration and response. The no-ADDLs control had a higher signal than expected, indicating high background. The high background in the no-ADDLs wells has been a significant difficulty in consistently obtaining data to the 1 fM range. Another obstacle has been preparing the detection probes to a sufficient concentration of 150 pM because much of the initial Au NP probe is lost in supernatant during the antibody conjugation and particle passivation.
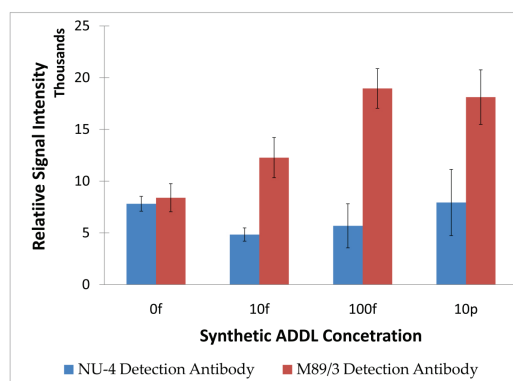

Figure 3. Determining the functionality of NU-4 as a detection antibody using NU-1 as the capture antibody. The NU-4 conjugation to the Au NP probes was conducted 2 days earlier than the M89/3 conjugation. While this may have impacted the results, it is also noteworthy that the NU-4 detection probes do not keep well. (± standard error of the mean (SEM), n=3)
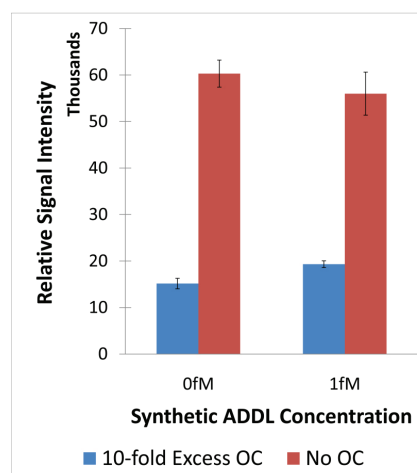
Figure 4. Oleocanthal treatments improve background. The + oleocanthal wells have substantially lower background than the no-oleocanthal wells ($\alpha = 0.05$, $P_{0fM} = 0.0009$, $P_{1fM} = 0.0191$). Similar results were noted with the 100-fold oleocanthal treatments. (± SEM, n=3)
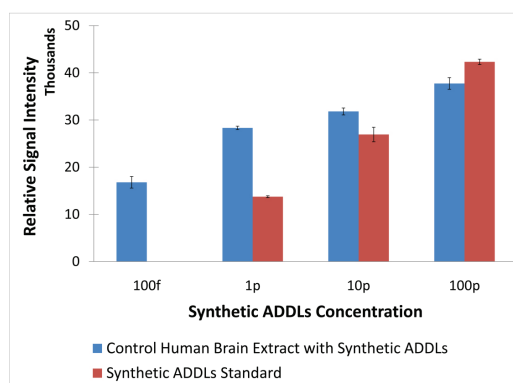
Figure 5. Control human brain extract with synthetic ADDLs. Similar results were noted with the control Tg mouse brain extract with synthetic ADDLs. (± SEM, n=3)
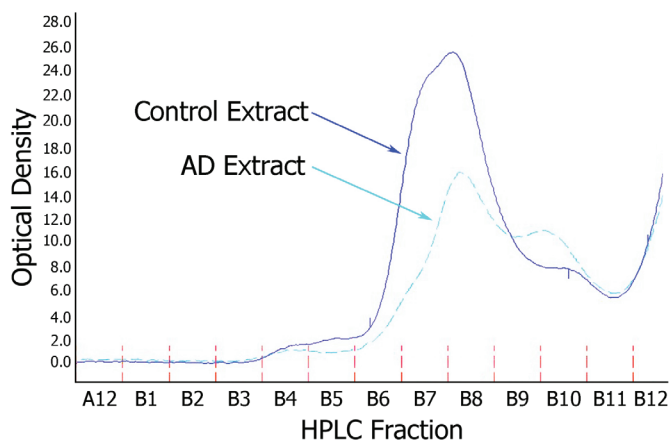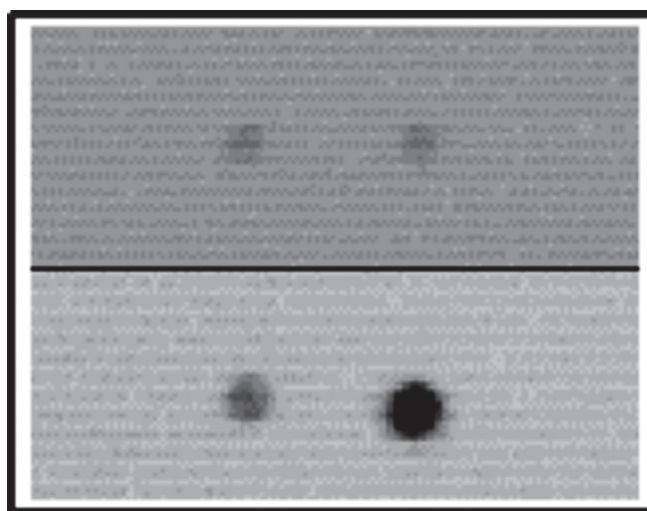
Figure 6. HPLC results for control and AD human brain extracts. Eighteen fractions of each extract were evaluated by dot blot, and 9 samples of each were evaluated by SIA. Region B9–B11 had elevated protein levels in the AD sample.


Figure 7. Dot-blot assays for control and AD human brain nonfractionated samples. The NU-1 dot blot (top) had faint signals for both nonfractionated samples. The other antibody dot blot (bottom) had distinct control and AD nonfractionated sample signals.

Determining the Functionality of NU-4 as a Detection Antibody

Once an ADDL-concentration-dependent response was generated using the M89/3, the functionality of NU-4 as a detection antibody was examined. The NU-4 antibody is monoclonal, unlike the polyclonal M89/3, and this set of experiments was conducted to determine if a monoclonal sandwich would have similar results to the monoclonal-polyclonal sandwich. As seen in Figure 3, both the M89/3 and the NU-4 had ADDL-concentration-dependent responses. However, the M89/3 had a higher signal strength and the NU-4 had a higher background shown by the 0fM well. These results are not conclusive, because other factors may have influenced the reduced NU-4 response. The NU-4 used in Figure 3 was conjugated to the Au NPs two days earlier than the M89/3. The age of the NU-4 probes may have resulted in degradation, which would impact the signal strength.

Oleocanthal Treatment for Improved ADDL Immunoreactivity

In order to improve ADDL detection and sensitivity, an oleocanthal (OC) treatment was conducted. OC is a component derived from olive oil and has previously been found to improve ADDL immunoreactivity.[10] Since OC has an impact on ADDL affinity toward antibodies, it may increase the sensitivity of the assay. However, the impact of the OC on the Au NP detection probes is also a consideration; thus, two experiments were run in parallel. One had an OC treatment only at the ADDL dilution stage, thereby only impacting the ADDL-capture antibody binding. The other had an OC treatment throughout the assay in the SIA buffer solution, thereby impacting both binding stages. A negative OC control was used for comparison, and the treatments were done in both 10-fold and 100-fold excess OC relative to the ADDL concentration. Figure 4 indicates that the OC treatment resulted in lower background in the lower concentration wells. Furthermore, the OC treatment did not have an apparent effect on the particles, with similar results for both treatments.

Synthetic ADDLs in Control Brain Extracts

To begin the transition toward biological samples, control transgenic (Tg) mouse brain extract and control human brain extract were tested with the standard 10-fold synthetic ADDL dilutions. This procedure would allow us to determine the impact of the biological extracts on the sensitivity of the assay and to modify assay parameters as needed. Rather than 60 μL depositions of the synthetic ADDLs, 50 μL were deposited with 10 μL of the extract. In both cases, the extract increased the signal response compared to the control, as seen in Figure 5. This result was not expected because previous work showed a decrease in signal for ADDLs in the presence of biological fluid, such as human plasma. The increased signal could be due to the low endogenous-ADDL concentration present in the control extracts.

HPLC-Fractionated Control and AD Human Brain Extracts

Control and AD human brain extracts were HPLC fractionated and examined by the dot-blot assay and by the SIA. Figure 6 has a segment of the HPLC chromatogram, in which region B9–B11 was of interest due to the elevated protein level in the AD sample. From these fractions, fraction numbers B3–B11 of each were analyzed by SIA, and fraction numbers A11–C4 of each were also analyzed by dot blot. Furthermore, the nonfractionated samples were also analyzed by each assay. For the dot-blot assay using NU-1, there were no signals for any of the fractions, and the nonfractionated samples had faint signals of a similar magnitude. However, in the dot-blot assay using a new higher-affinity antibody, there were distinct signals for the nonfractionated samples, and no signals for any fractions (Figure 7). The SIA signal was four-fold greater for the AD nonfractionated sample compared to the control nonfractionated samples (Figures 8,9). Significantly, the SIA was also able to detect all of the fractionated samples, and in the region of interest for fractions B9–B11 there was an elevated signal in the AD extract (Figure 9). Thus, the SIA was able to detect with greater sensitivity using NU-1 than the dot-blot assay. It would be of interest to use the SIA with the higher-affinity antibody for greater sensitivity due to the initial results from the dot blot.

Control Human Brain Extract

1pM | B9 | B10 | B11 | NF

PSA | B9 | B10 | B11 | NF

AD Human Brain Extract
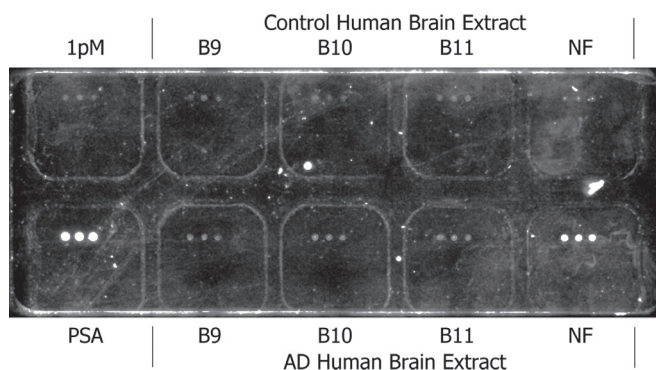
Figure 8. Verigene slide image for SIA for control and AD human brain HPLC fractions. The PSA well is a positive assay control, while the 1 pM well is a synthetic ADDLs control for normalization across different slides. Fractions B9–B11 have higher signals in the AD sample. Furthermore, the nonfractionated AD sample is saturated. The analysis of this data is in Figure 9.
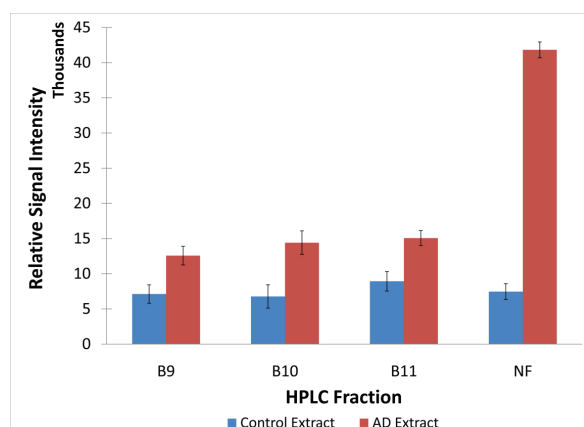
Figure 9. SIA for control and AD human brain HPLC fractions. The SIA was conducted using NU-1 and M89/3. It was able to detect signals across all 9 fractions for both the control and AD samples. Furthermore, in the B9–B11 region there was elevated signal intensity for the AD samples, corresponding to the peak in Figure 6. The SIA was able to detect a statistical difference in signal intensity of nonfractionated samples ($\alpha = 0.05$, P = 2.46E-5), with a four-fold difference in magnitude, while the dot blot in Figure 7 has faint signals when using NU-1. (± SEM, n=3)

Conclusions

Ultrasensitive detection of a pathogenic biomarker for AD, such as ADDLs, is relevant in developing a definitive premortem diagnostic tool. Nanotechnology-based techniques for sensitive protein detection, such as the SIA, are a significant step forward in determining biomarker concentrations. In this study, the SIA was further developed for use in measuring ADDL concentrations. Initially, synthetic ADDLs were analyzed in the assay buffer; however, progress was made toward Tg mouse brain extracts and human brain extracts. Initially, an ADDL-concentration-dependent dose response was determined using an M89/3 detection antibody. Then, the functionality of NU-4 as a detection antibody was examined. Oleocanthal treatments were utilized to improve the assay sensitivity by reducing the background in the 0 fM wells. Lastly, the SIA was conducted on 9 HPLC fractions of each

control and AD human brain extracts. When analyzed by dot blot using NU-1 there were no signals; however, not only was the SIA able to detect signals in each fraction, it was also able to distinguish between the control and AD samples. Thus, the SIA is promising for detecting ADDLs in biological samples and may have improved sensitivity using recently developed higher-affinity antibodies.

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