Characterization of Anti-ADDL Antibodies for Use in Tandem LSPR-MALDI Investigation of Alzheimer's Disease Brain Extracts

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Abstract

Small, soluble, self-assembling peptide Aβ-oligomers, known as ADDLs (amyloid-derived diffusible ligands), accumulate in the Alzheimer's disease (AD) brain as Aβ 12-mers and are thought to contribute to AD neuropathological conditions. Unlike plaques, which were once thought to cause neuronal death due to insoluble amyloid plaque deposits, ADDLs initiate abnormal changes in dendritic spine morphology and receptor trafficking, ultimately leading to memory failure. Due to this significant brain impairment caused by AD, there is a great demand for diagnostic techniques to identify the toxic oligomers and assess their interactions with various antibodies. Anti-ADDL antibodies have prevented ADDL toxicity in vitro, and, with the use of dot and Western blots, the antibody with greatest ADDL affinity and specificity has been characterized. This maximum affinity and specificity is necessary for Matrix Assisted Localized Desorption Ionization (MALDI), a mass spectrometry (MS) technique that allows measurement of toxin mass and concentration after identification of the toxic oligomer molecule. Along with the ADDL based bio-barcode, the localized surface plasmon resonance (LSPR) spectroscopy diagnostic assay may allow for a comparison of ADDL concentrations in cerebral spinal fluid (CSF) between control patients and patients with AD symptoms, perhaps acting as a sensor that may lead to earlier therapeutics for AD patients.

Introduction

Diagnostic techniques for Alzheimer's disease are being developed with the aim of exposing samples of human brain extracts and CSF to high-affinity antibodies that bind toxic ADDLs oligomers. For the continued development of such techniques, it is essential to identify exactly what ADDLs comprise and what interactions they have with various antibodies. This research focused on three goals: characterizing antibodies using dot and Western blots in order to find the antibody with greatest ADDL affinity; running the LSPR diagnostic assay using the ADDL-antibody complex with the greatest binding affinity; and using MALDI-MS to obtain information about toxin mass and concentration.

To properly evaluate harmful effects of the oligomer molecules, synthetic toxic ADDLs have been prepared and are being tested on rat hippocampal neurons using monoclonal antibodies.[1] Synthetic oligomers are used because they show structural similarity to brain-derived oligomers with respect to "mass, isoelectric point, and recognition by conformation-sensitive antibodies."[2] In addition, both types of oligomers display similar attachment patterns to hippocampal neurons and comparable binding on dendrite surfaces in small bundles with ligand-like specificity.[2] Antibodies against synthetic ADDLs, created by the Klein laboratory, have successfully demonstrated that these antibodies prevent ADDL toxicity in vitro.[1] It has also been shown that these antibodies prevent synapse loss, AD-type hyperphosphorylation, and generation of ROS in vitro. Use of antibodies against synthetic Aβ-oligomers is a valuable way to assess the characteristics of the toxins and eventually design a therapeutic mechanism to treat affected patients. Once it becomes feasible to identify exactly what binds to the antibodies, it will be possible to recognize distinctive properties of the oligomers.

In addition to the bio-barcode assay, LSPR spectroscopy — a specific form of surface plasmon resonance (SPR) — coupled with MALDI-MS may be utilized as a diagnostic technique. LSPR, a nanosensor that uses a concentrated beam of visible light to interact with nanoparticles, causes absorption and scattering. In addition, LSPR makes use of silver nanoparticles marked with anti-ADDL antibodies to detect very low concentrations of ADDLs. This assay will also be able to measure the thermodynamics and kinetics of binding.[3] MALDI-MS, a complementary analytical technique, will allow measurement of the toxin mass and ultimately identification of the toxic oligomer molecule. Future development of the bio-barcode and LSPR diagnostic assays will allow physicians to retrieve CSF from patients with AD symptoms, compare ADDL concentration between the sample and the control patient, and possibly diagnose patients with AD.

Background

Increasing evidence suggests that plaques do not actually initiate the neurological conditions associated with AD.[4] Tangles, which are made up of hyperphosphorylated tau, must be present in order for Aβ to induce the degeneration of brain cells.[5] In fact, experiments aimed at preventing Aβ aggregation led to the discovery of small soluble Aβ-oligomers as the toxin rather than the fibrillar Aβ-derived previously thought to contribute to AD neuropathological conditions. Experiments by Finch and colleagues[6] based on the interaction of Aβ and ApoJ (clusterin), an up-regulated, plaque-associated molecule in AD brains, suggested that neurological damage in the AD brain may be the result of soluble Aβ-oligomers.[7]

ApoJ successfully blocked Aβ from forming large aggregates that encouraged plaque formation. Instead, in the presence of ApoJ, Aβ produced toxic Aβ-oligomers that are not large fibrillar aggregates but small globular molecules with nanometer diameters.[7] Oligomers,

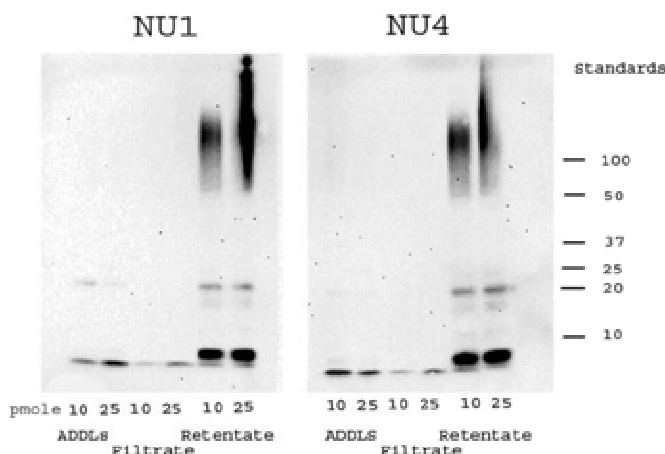

Figure 1. Western blot showing binding of primary antibodies NU1 and NU4 to ADDLs, filtrate, and retentate at different concentrations. ADDLs incubated with NU1 and NU4 show monomer, but only ADDLs incubated with NU1 shows tetramer. Filtrate incubated with either antibody shows only monomer at both 10 and 25 picomole concentrations. Retentate samples incubated with NU1 and NU4 both show a clear signal for monomer, dimer, trimer, tetramer, and 12-24mer.

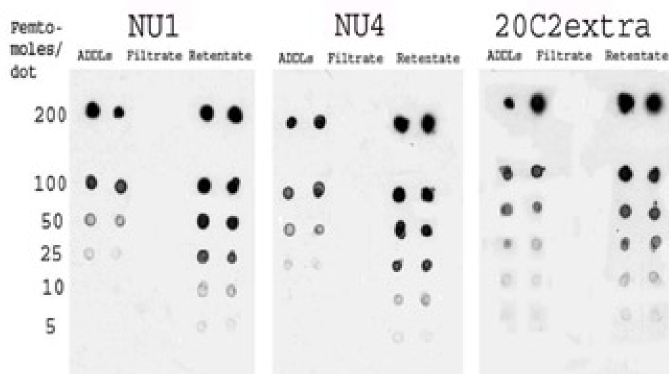

Figure 2. Dot blot shows binding affinity of primary antibodies NU1, NU4, and 20C2extra to ADDLs, filtrate, and retentate at different concentrations. ADDLs incubated in 20C2extra visualize the 5 and 10 femtomole dots, while those incubated in NU1 and NU4 do not show such dots. Filtrate incubated with either of the three antibodies does not show any binding. Retentate with NU1, NU4, and 20C2extra all seem to visualize equivalent intensities.

known as ADDLs, for pathogenic Aβ-derived diffusible ligands, accumulate in the AD brain as Aβ 12-mers (~54k Da) and are the essential disease-defining molecules.[7] Not only did this suggest that ADDLs are self-assembling peptide nanoparticles,[8] but it also verified that fibrils are not the only toxins derived from Aβ.[7] This theory was developed using tg-mouse AD models, since similar oligomers were present in mice that display memory trouble and recollection letdown.[9]

ADDLs harm the neuronal synapse, the primary information pathway by which neurons communicate. By binding to postsynaptic sites on the synapse, ADDLs cause abnormal changes in dendritic spine morphology and receptor trafficking, ultimately leading to memory failure.[10] ADDLs, highly specific gain-of-function ligands, bind to specific synapses and lead to increased expression of the memory-linked gene Arc. The protein Arc (activity-regulated cytoskeletal protein) is the resultant product of an intermediate early gene necessary for long-term memory formation.[7] Arc is ectopically stimulated when neurons are introduced to ADDLs, suggesting that ADDLs cause Arc overexpression, which inevitably leads to learning dysfunction.[7]

In tg-Arc mouse models, the overexpression of Arc seems to be associated with memory failure by causing loss of NMDA cell-surface receptors and altering synaptic plasticity. Since ADDL binding sites are near NMDA receptors, antibodies against these receptors diminish ADDL binding and restrain ADDL-stimulated reactive oxygen species (ROS) formation, which occurs upon binding.[7] "The net neurological effect of oligomers in the hippocampus thus is to repress positive synaptic feedback."[7] Since memories are thought to be characterized by highly interconnected networks of synapses in the brain, loss of synaptic plasticity may in fact distress one of the most essential neurochemical foundations of learning and memory.

Approach

The primary goal of this research project was to characterize antibodies using dot and Western blots in order to obtain an LSPR and MALDI signal when antibodies and ADDLs were annealed to the surface of silver nanoparticles. Dot and Western blots were used to determine which antibody bound the ADDLs with better affinity and specificity before proceeding with LSPR and MALDI analysis. Upon completion of the blotting techniques, the affinity of antibody binding to ADDLs was analyzed by intensity of sample staining and the best antibody was chosen. After silver nanoparticles were placed on the surface of a small round disk and a shift in the LSPR signal was obtained, antibodies, followed by ADDLs, were annealed to the surface of the nanoparticles and an LSPR shift was obtained after each addition. The shift in the LSPR signal determined if the antibody had bound to the ADDLs.

Determining which antibody bound ADDLs with higher affinity and specificity was crucial because the ultimate goal was to test Aβ-oligomers found in CSF using MALDI-MS as a diagnostic tool. In CSF, there may be molecules with molecular weights similar to ADDLs, and if antibody binding is not specific, signals from undesired molecules may be obtained. The MALDI spectrometer was then used to analyze the antibody-ADDL complex. MALDI-MS provided information regarding the mass and concentration of the molecules. Mass is extremely important because once the size of the toxic ADDL is known, development of therapeutics to treat Alzheimer's disease is more effective.

Dot Blot Technique

One of the preliminary evaluation methods of antibody affinity was the dot blot procedure. This spotting technique used a mixture containing the molecules to be detected in solution. The samples were applied directly, as a dot, onto a membrane known as nitrocellulose paper. Although this method offered no information regarding size of the biomolecule, it did suggest information about its presence or absence. In addition, two solutions of fractionated ADDLs were used; the fractionated filtrate contained an ADDL mixture of 50 kD or less, while the fractionated retentate contained ADDLs greater than 50 kD. In the experiment, ADDLs and the two fractionated solutions of micromolar concentrations were diluted via serial dilution in F12 to six concentrations ranging from 200 nm to 5 nm.

After each dilution was made, a 1μl sample was spotted onto nitrocellulose paper, using a template and lightbox. After all dots were spotted in duplicate or triplicate, the nitrocellulose was allowed to dry for 15 min after the last spotting. The membrane was then blocked in 5% nonfat dry milk (NDM) in tris-buffered saline (TBST) composed of 20 mM Tris-HCl, 200 mM NaCl, and 0.1% Tween 20 at pH 7.6 for

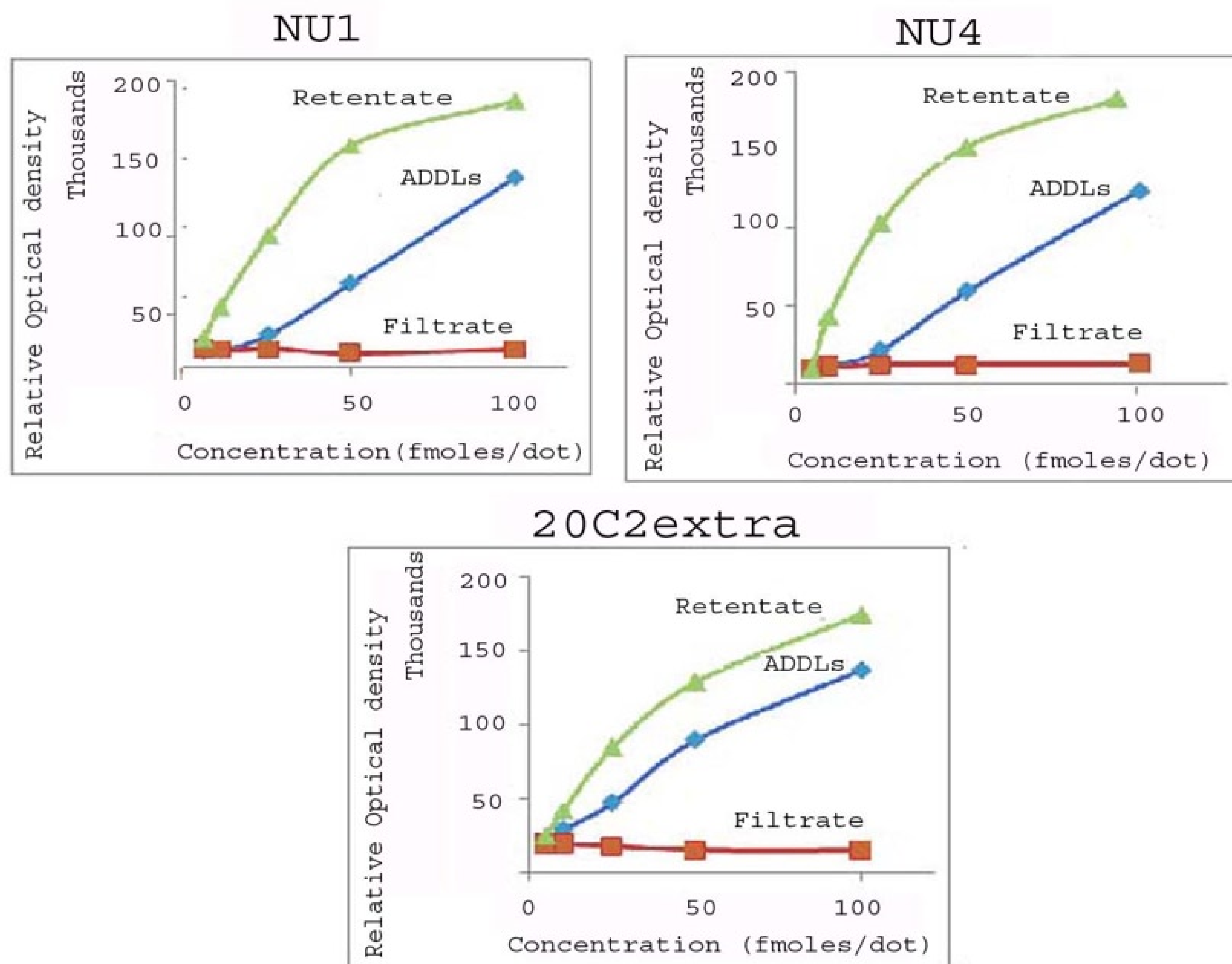

Figure 3. Quantified dot blot results show binding affinity for each of the primary antibodies NU1, NU4, and 20C2 extra to ADDLs, filtrate, and retentate at different concentrations.

1 hr at room temperature on a shaker or overnight in the cold room. Next, primary antibodies NU1, NU4, and 20C2extra (1mg/ml) were diluted in blocking buffer to varying concentrations, between 0.125 μg/ml and 1μg/ml. The ADDL, filtrate, and retentate dots spotted on the nitrocellulose were then incubated in each of the primary antibodies in order to determine the optimal antibody binding concentration for ADDLs, filtrate, and retentate solutions. After 1.5 hr, the samples were washed in TBST three times for 15 min and then incubated in secondary antibody. All samples were incubated together using HRP conjugated anti-mouse IgG secondary antibody diluted 1:20,000 for one hr with shaking at room temperature. The samples were then washed in TBST three times for 15 min and incubated with half-strength SuperSignal before being exposed to film.

Western Blot Technique

Used as a method for detecting specific proteins in a sample, the Western blot aided in the preliminary determination of the strongest binding antibody. The Western blot used gel electrophoresis to separate ADDL, filtrate, and retentate samples based on differences in charge and size, and determined whether dimer, trimer, or 12-24mer of 10 and 25 pmoles were present and to which antibody they bound with greatest affinity. ADDLs, filtrate, and retentate of 54.6 μM, 42.8 μM, and 36.4 μM initial concentrations, respectively, were separated using a Tris-Tricine SDS gel. Sample concentrations were 10 and 25 pmole ADDL, filtrate, or retentate per lane. After running the gel at 125V until the tracking dye reached the bottom (approximately 1.5 hr), the gel was removed and samples transferred to nitrocellulose in TBST plus 0.02% SDS in the cold at 100V for one hr. The membrane was then removed and cut into separate samples so that each set was independently incubated. A different primary antibody (1 μg/ml) was added to each sample in blocking buffer for 1.5 hr at room temperature. Samples were then washed three times for 15 min after which they were incubated in secondary antibody. All samples were incubated together using HRP conjugated anti-mouse IgG secondary antibody diluted to 1:40,000 for 1 hr shaking at room temperature. The samples were then

washed in TBST three times for 15 min and incubated with half-strength SuperSignal before being exposed to the Kodak machine for imaging.

LSPR Spectroscopy and MALDI-MS Techniques
Silver nanoparticles, through a deposition system, were annealed to the surface of a glass disk. This process, known as nanosphere lithography (NSL), is a nanofabrication technique capable of producing a large range of nanoparticle structures and well-organized nanoparticle arrays. Polystyrene nanospheres were deposited onto the surface of the glass disk, forming a sphere mask with evenly spaced gaps. After the disk was inverted and the gaps were filled with metallic silver, the polystyrene was removed while the silver nanoparticles remained on the glass disk. This disk was then put into self-assembling monolayer (SAM) of 11-mercaptoundecanoic acid/1-octane-thiol, which bound to the nanoparticles. The COOH group of the SAM interacted with the N-terminus of the antibody, forming a bond. A control signal was obtained from the nanoparticles in phosphate buffered saline (PBS).

Antibodies in PBS were then inserted into the LSPR. If the antibodies bound to the SAM and the nanoparticles, a red shift was observed. ADDLs in PBS were then inserted, causing another red shift, indicating another molecule had bound. Adding the antibodies and ADDLs caused a red shift in the extinction wavelength, meaning that the device could act as a sensor. Once the LSPR provided the verification that the bound molecules were ADDLs, the MALDI-MS was used and provided information regarding the mass of the bound molecules.

Results and Discussion
Western blots were used to determine which ADDLs species bound to the antibody. As shown in Figure 1, mononer, dimer, trimer, tetramer, and 12-24mer were observed in different ratios with ADDLs, filtrate, and retentate at varying concentrations. ADDLs incubated with NU1 and NU4 showed the monomer, but only ADDLs incubated with NU1 showed the tetramer. This result suggests that NU1 has greater specificity for the ADDL tetramer. Filtrate incubated with either antibody shows only the monomer at both the 10 and 25 picomole concentrations. This result shows that filtrate species greater than the monomer have no affinity for either antibody. Retentate samples incubated with NU1 and NU4 both show a clear signal for the monomer, dimer, trimer, tetramer, and 12-24mer. These data reveal that both primary antibodies have similar specificity for the retentate samples while both showing preference for the 12-24mer in the retentate. Knowing whether the 12-24mer is present in the sample is significant because a 12-24mer signifies the presence of a toxic species molecule greater than 50 kD in size.

Dot blots were used to determine the affinity of different antibodies to ADDLs. When the ADDLs, filtrate, and retentate solutions at different concentrations were spotted onto nitrocellulose paper, incubated with antibody, and exposed to film, the appearance of dark dots provided information regarding affinity of antibody binding to ADDL, filtrate, or retentate. Figure 2 shows the appearance of the 10 and 5 femtomole dots of ADDLs incubated with 20C2extra and the absence of these dots for ADDLs incubated in NU1 and NU4, suggesting that primary antibody 20C2extra has greater affinity for ADDLs as compared with NU1 and NU4. ADDLs incubated with NU1 and NU4 have a similar appearance, indicating that antibody binding may be of equivalent affinity. Based on the dot blot seen in Figure 2, none of the three primary antibodies has binding affinity to filtrate samples, as indicated by the absence of dots specifying binding of antibody to any filtrate. Retentate, on the other hand, seems to have

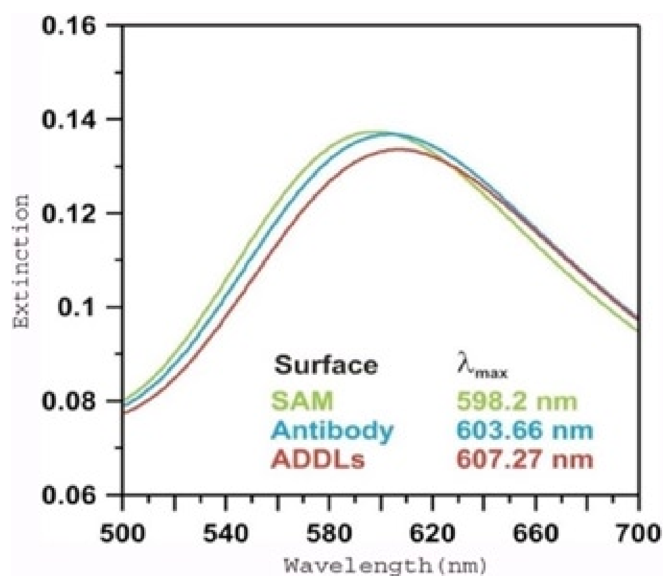

Figure 4. LSPR spectra for each step of the preparation of the silver nanosensor at low concentrations of ADDLs. This trial used 50 nm Ag on 390 nm spheres, 250 nm NU1, and 500 nm ADDLs. The green curve represents the SAM solution that bound to the surface of the nanoparticle, while the blue curve represents the addition of primary antibody NU1 and its binding to the surface of SAM. Finally, the red curve indicates the addition of ADDLs and its binding to the antibody. Observing the plot of extinction vs. wavelength, it is obvious that a red shift occurs after the addition of each molecule, indicating specificity of binding.

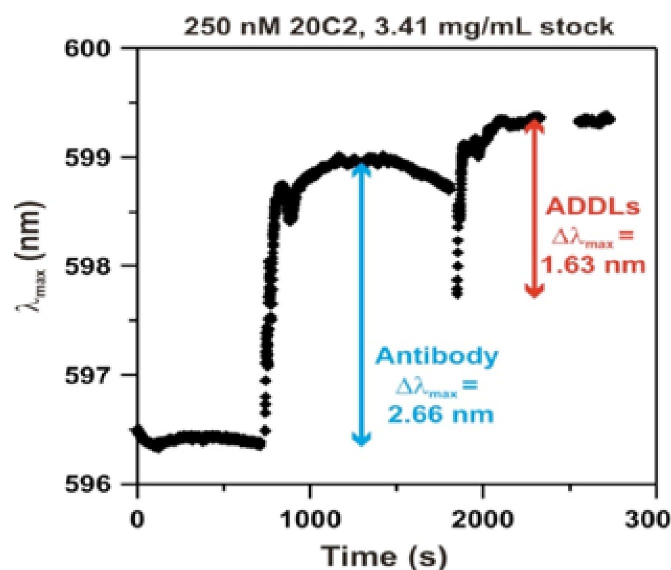

Figure 5. LSPR real-time data showing wavelength vs. time. This plot shows, in sec, the amount of time taken for each molecule to bind to the layer of molecules added before it. The first section of the curve, from 0 to 800 sec, shows PBS buffer in the LSPR. At approximately 800 sec, antibody is added, and a red shift is observed from 800 sec to 1800 sec. Finally, at 1800 sec, ADDLs are added, and another red shift is observed from 1800 sec until 2800 sec.

high binding affinity to all three primary antibodies. The similar affinity of binding for retentate samples is indicated by equivalent dot intensity for all three antibodies incubated with retentate. These data are quantified in Figure 3. In summation, retentate has the greatest affinity of binding, followed by ADDLs, and lastly filtrate, which has no binding affinity to any of the three antibodies.

Both the Western and dot blots offered evidence that all three antibodies bind retentate with strongest affinity and specificity, followed by ADDLs and lastly by filtrate. This conclusion was reached after studying the data provided in Figures 1 and 2 and observing the intensity of staining for these two blots. Because the data seem relatively similar for the antibodies, to differentiate with certainty the antibody with greatest ADDL affinity and specificity was difficult. It was still possible to choose a representative antibody to utilize with the LSPR nanotechnology technique.

LSPR was used to determine whether specific binding was occurring between ADDLs and antibodies. After silver nanoparticles were annealed to the surface of a glass disk and placed in SAM solution, antibodies plus ADDLs were added sequentially. Figure 4 shows that a red shift in wavelength occurred after the addition of each solution. Silver nanoparticles placed in SAM were used as the base point, and a wavelength of 598.2 nm was obtained. After the antibody was added, another red shift occurred and a wavelength of 603.66 nm was observed. This red shift indicated that the antibodies bound to the SAM on top of the nanoparticles. After verifying that the antibody had bound, ADDLs were added, and another red shift of 607.27 nm was obtained; this was a true indication that ADDLs were binding antibodies with specificity.

The real time data, Figure 5, obtained from the LSPR shows a plot of wavelength (nm) vs. time (seconds). This image shows the amount of time antibodies took to bind to SAM (on the surface of the nanoparticles) and the time ADDLs took to bind to the surface of the antibody. In addition, each molecule made a distinctly shaped curve characteristic of that molecule's binding. The apparent red shift seen after the addition of antibody followed by ADDLs allows conclusions to be drawn that validate the specific binding of ADDLs to antibodies.

Previous work using LSPR to test ADDL-antibody binding used a different order of molecule addition. Earlier LSPR experiments annealed the silver nanoparticle to the surface of a glass disk and placed it in SAM solution, similar to the method used in this experiment. The difference, however, is that earlier studies placed ADDLs first, obtained a red shift, placed the antibody on top of the ADDLs, and still obtained a red shift. Future practical clinical application of the LSPR has encouraged changing the order of molecule addition, because it is believed that when antibodies are placed first followed by ADDLs, there is greater specificity of binding. Since the particular composition of CSF is not known exactly, placing the antibody first provides a means of obtaining a more accurate signal. It is reasonable to imagine a diagnostic tool that has nanoparticles attached first and then antibodies. Once CSF is obtained, it could be placed across the antibody surface and provide a means for specific ADDL-antibody binding.

Although the MALDI-MS was not run on the sample used in LSPR spectroscopy above, analysis of previously obtained data suggests that this technique would be effective at measuring the mass of ADDLs bound to the antibodies. Earlier data obtained from the Van Duyne laboratory has shown the appearance of the monomer, dimer, trimer, and 12-24mer of ADDLs. Further experimentation using LSPR-MALDI should make it possible to assess the distinct size of the sought-after ADDL.

Conclusion

The interaction of ADDLs with various antibodies is essential for the future development of techniques capable of detecting the disease-causing molecules in individuals with Alzheimer's disease in hopes of designing therapeutics to prevent further AD damage. This antibody characterization was possible through the use of dot and Western blots; these techniques assessed the interactions of ADDLs with various antibodies for future use in LSPR-MALDI analysis. It is necessary to continue characterizing the antibody with greatest ADDL affinity and specificity in order to obtain more precise and informative LSPR-MALDI measurements. As MALDI data collection continues, and more information is obtained about size and concentration of ADDLs in a sample, CSF may ultimately be used to compare control patients and AD patients. The data obtained from this study's LSPR experiment confirmed that the use of silver nanoparticles is effective in studying binding affinity. The addition of antibodies followed by ADDLs to the surface of silver nanoparticles may provide an efficient clinical diagnosis technique.

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