Nanotechnology-Based Approaches to Alzheimer's Clinical Diagnostics

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Abstract

The bio-barcode assay is a nanotechnology-based method for capturing and detecting proteins. It shows extraordinary sensitivity in detecting certain antigens at extremely low concentrations, now without the use of the polymerase chain reaction (PCR). This assay uses disease identification biomarkers that cannot be used in the conventional assays of today. ADDLs are considered neurotoxins in Alzheimer's disease (AD) pathogenesis. This discovery has led to many different methods to detect ADDLs in AD-diagnosed patients. One approach is to detect ADDLs in human plasma, which is more widely used clinically and easier to obtain than CSF. Here we will attempt to detect ADDLs in plasma samples from 16 patients — 8 with AD and 8 without the disease. The results show that many different protocols and methods must be employed to successfully detect ADDLs using the bio-barcode assay. While an efficient protocol must still be developed, this study helps to establish a new diagnostic approach for AD using the bio-barcode assay.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease for which there is neither a cure nor a good clinical diagnostic.[1] AD is the leading cause of dementia in persons over the age of 65 and affects more than 4 million Americans. Many genetic, biochemical, and animal studies strongly suggest that the amyloid β peptide plays a significant role in the pathogenesis of AD. The amyloid β peptide is a 42 amino acid peptide that was first discovered as the monomeric subunit of large, insoluble amyloid fibrils of a type of plaque found in Alzheimer's disease. The amyloid β peptide has been known to accumulate into small soluble oligomers called ADDLs. ADDLs are amyloid-derived diffusible ligands that are considered neurotoxins in Alzheimer's patients and are thought to cause neurological problems relevant to memory.[2]

Despite the discovery of ADDLs, no clear or distinct diagnostic for AD has emerged. Two major approaches have been tried for AD diagnosis. One method is based on measuring the total tau protein or amyloid β protein concentration in the cerebrospinal fluid or plasma. This approach has led to inconclusive results. The other approach targets only suspected pathogenic markers such as cleaved tau protein, phosphorylated tau protein, or ADDLs. Again, the accuracy of the results is questionable, in part because concentrations of such markers in cerebrospinal fluid are so low in the early stages of AD that they cannot be identified accurately with the conventional ELISA or blotting assay. Therefore, steps have been taken recently in order to detect Alzheimer's disease with more accuracy by using a nanotechnology-based approach. Since its invention, the bio-barcode assay has become a powerful investigative tool for detection of both protein and nucleic acid targets.[1]

Background

In a previous report the ultrasensitive nanoparticle-based bio-barcode assay was used to determine the approximate ADDL concentration in cerebrospinal fluid taken from 30 subjects, 15 of whom were diagnosed with AD through postmortem analysis of the brain, and 15 of whom did not have the disease.[1] Because its extraordinary sensitivity allows proteins to be detected at very low concentrations, the bio-barcode assay gives rise to new approaches to detecting AD.[1] Here we will demonstrate that ADDLs may serve as a target for detection. The results suggest that using plasma instead of cerebrospinal fluid could prove to be a successful way of developing a diagnostic assay for AD based upon detection of ADDLs.

The key to the bio-barcode assay is the homogeneous separation of specific antigens by means of a sandwich process involving DNA modified gold (Au) nanoparticles and magnetic micro particles (MMPs).[2] The particles are functionalized with (Abs) antibodies that are specific to the antigen of interest. In this case the antigen of interest is the ADDL. The increased sensitivity of this assay derives mainly from the effective appropriation of antigen, and the amplification process results from the release of numerous barcode DNA strands from each antigen recognition and binding event.[1] The barcode DNA strands are detected using the scanometric approach, resulting in detection limits as low as 500 zM (10 strands in solution).[3]

Approach

The bio-barcode assay is used to detect ADDLs in samples of human plasma. Detecting ADDLs in plasma could be an alternative to current assays such as PCR or ELIZA, which lack the high sensitivity of the bio-barcode assay.[4] The first step is the functionalization of gold (Au) nanoparticles with DNA and Abs, MMPs with Abs, and glass slides spotted with complementary strands of DNA.

Functionalization of 30 nm Gold Particles
The 30 nm diameter Au particles were prepared by using 1 ml of Au colloid that was functionalized with 1 ug of (M90) polyclonal antibodies. 30 nm Au particles were used during the detection step instead of 13 nm Au particles to increase the amount of detectable barcode DNA.[3] The M90 is a polyclonal antibody that recognizes the specific protein of interest, which is the ADDL. Also, the Au nanoparticle has to be adjusted to a pH of 9 by adding 3.62 ul of NaOH. The particles were modified with DNA by soaking the DNA in dithiothreitol (DTT) (cytology reagent) solution for two hours prior to adding it to the Au colloid. The DNA was immersed in DTT in order to cleave the disulfide on the DNA. One O.D. of DNA was added to 500 ul of DTT solution to ensure the use of all the DNA strands that serve as an amplification target.[1] O.D. — optical density — is the absorbance of an optical element for a given wavelength λ per unit distance. We had to make certain that we had 1 O.D. of DNA. We used a UV-spectrometer to measure the absorbance of light at 260 nm. The DNA and Abs were then added to the Au colloid. A salting protocol was then performed after an 18-hour waiting period. The purpose of the salting protocol is to ensure that the thiol group from the DNA does not displace

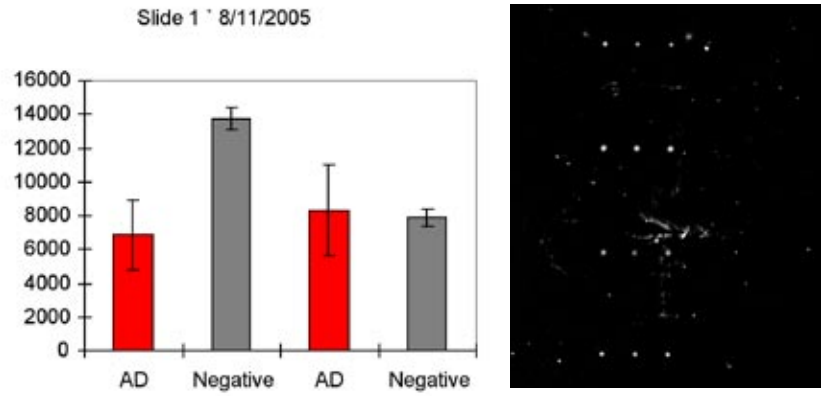

Figure 1a: Only one in four AD patients has a higher intensity than the control (negative) patients on this slide. Image on right represents graph: AD = first and third rows from top; negative = second and fourth rows from top.

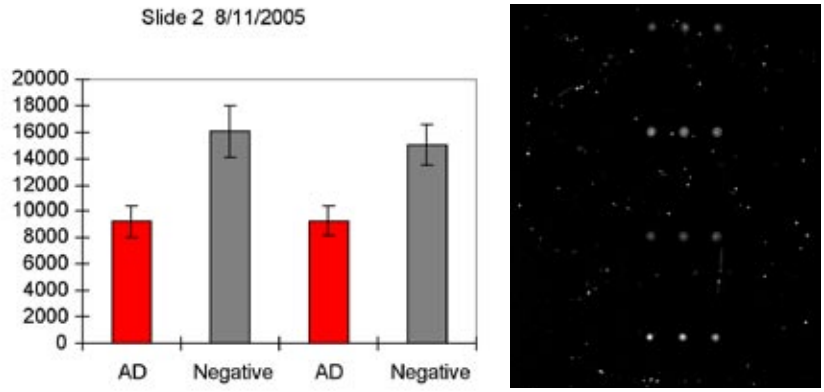

Figure 1b: All negative control patients have a higher intensity than AD patients. Image on right represents graph: AD = first and third rows from top; negative = second and fourth rows from top.

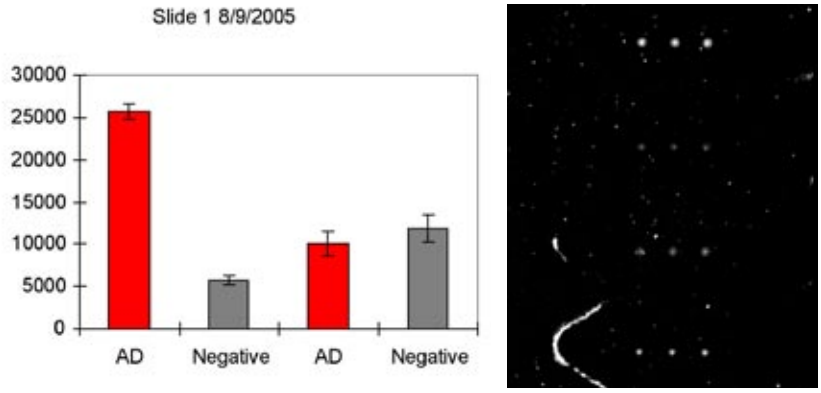

Figure 1c: Only one in four AD patients has a higher intensity than control (negative) patients on this slide. Image on right represents graph: AD = first and third rows from top; negative = second and fourth rows from top.

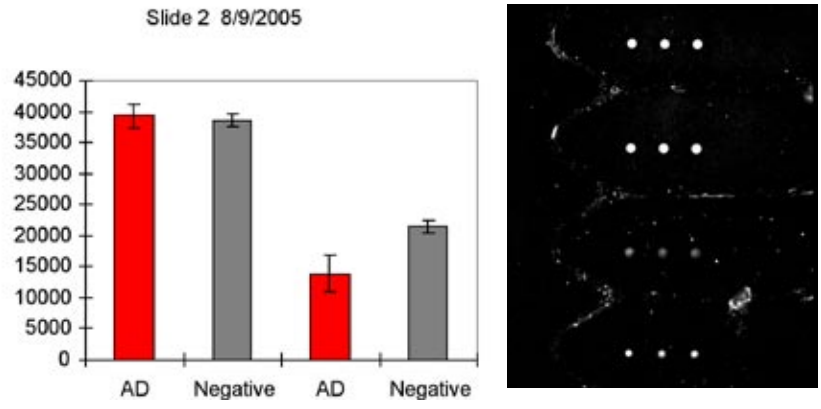

Figure 1d: Only one in four AD patients has a higher intensity than control (negative) patients on this slide. Image on right represents graph: AD = first and third rows from top; negative = second and fourth rows from top.

the citrate surface of the Au nanoparticle. In addition, the salting protocol ensures that the DNA is straightened and appropriated correctly around the surface of the Au particle. This was done by slowly adding the right amount of NaCl to the Au nanoparticle to guarantee DNA surface binding. Before the salting protocol the Au nanoparticle was brought to final concentration of .01M-phosphate buffer and 2M NaCl. Either of the equations below can calculate the amount of phosphate buffer (PBS) and NaCl to add to the Au colloid in order to stabilize the colloid to 0.2 NaCl. Finally, after the unknown final concentrations were found, the colloids were brought to 0.01% tween 20, using 10% tween 20 calculated as indicated in the third equation below. Tween 20 is a nonionic surfactant that effectively suppresses unspecific reactions between antibodies, antigens, and other molecules.

First Equation
$M_1V_1 = M_2V_2$

M_1 = Initial total concentration of PBS or NaCl of Au colloid

V_1 = Initial total volume of Au colloid

M_2 = Final concentration of Au colloid

V_2 = Initial total volume of colloid + X or Y of unknown value

X = volume of 100 mm phosphate buffer solution pH 7.4

Y = volume of 2 m NaCl in water

Second Equation
0.1x/(x+y+initial volume of colloid) = 0.01 (desired final phosphate concentration is 10 mM)

2y/(x+y+initial volume of colloid) = 0.2 (desired final salt concentration is .2M)

Third Equation
Total volume of Au colloid + (final phosphate buffer concentration found) = the amount of Tween 20 that has to be added to Au colloid with the final phosphate buffer concentration found in the second equation.

Functionalization of Magnetic Microparticles (MMPs)
Uniform µm tosylactivated magnetic beads (Dynabeads® M-280, Dynal) were modified with 31 µm of $20C_2$ of (mAbs) monoclonal antibodies specific to ADDLs, according to the manufacturer's protocol.

Glass Slides Spotted with DNA
The glass slides were spotted with modified half-complementary DNA strands by using a microarrayer according to a standard published procedure.

Preparing the Barcodes for Use in the Assay
In a typical assay 10 ul of Au particles functionalized with Abs and DNA were added to eight samples of ADDLs, which were already bound to 30 ul of MMPs. The particles were washed repeatedly with (PBS +1% Tween 20 and 5% formamide). The significance of the 5% formamide is to guarantee that the MMPs and the Au particles do not stick together during the sandwich complex. Also, .1mg/ml of BSA was added to make sure that specific binding of the sandwich complex was occurring during the incubation period. The process of the sandwich complex involves the Au particles functionalized with Abs and DNA and the MMPs functionalized with Abs. Both of these particles bind to ADDL, causing the ADDL to become sandwiched between the two particles. During the washing of the sandwich complex, the complex was very gently vortexed so as not to disrupt it. Repeated washing of the sandwich complex was necessary to remove any unbound Au particles. We then added 50 ul of 10 mM DTT solution to the particles in order to cleave the DNA from the Au particles that successfully sandwiched the ADDL. When the DTT solution reacted with the DNA, the DNA was released, causing the dehybridization of the sandwich complex, which resulted in barcodes. Once the Au particles and MMPs successfully sandwiched the ADDL, the MMPs were then isolated or immobilized by a magnetic separator, and the remaining unbound antigens were removed by repeated washing with (PBS + .1% Tween 20 + 5% formamide for 3 times and 2 times with formamide). The particles were centrifuged, and the supernatant containing the cleaved DNA or barcodes was used in the bio-barcode assay.

Running the Assay
The next steps required a spotted slide containing oligonucleotides that were complementary to half of the barcode DNA sequence. Glass slides or chips already spotted with complementary DNA were removed from a desiccator apparatus. Before the spotted slides could be used, we added (1 X SSC Rehydration) fluid to the chip.

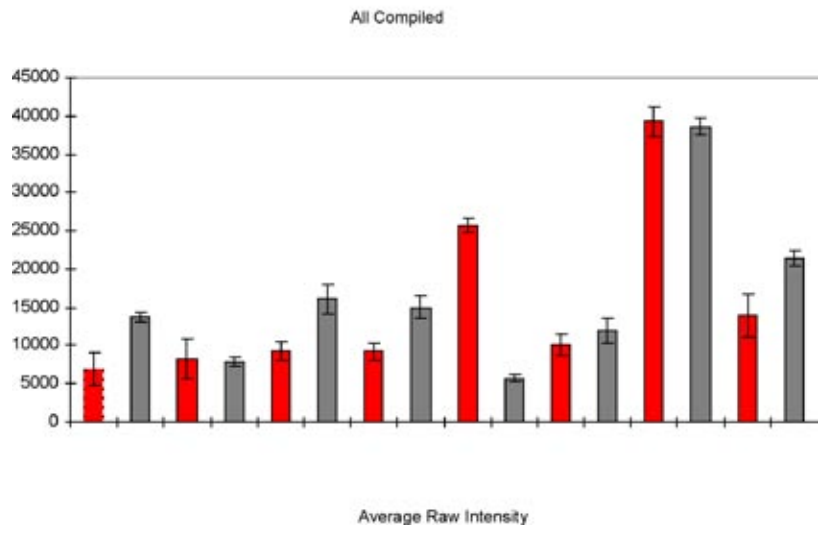

Figure 2: All slide figures (1a,b,c,d) are compiled into one chart to show overall average raw intensity.

This solution contains some citrate, making the chip ready for use. Next, the slide was spun slightly dry, and a clean gasket was put on the slide. To each of the gasket's four wells 16 ul of barcodes was added. The slides then underwent incubation for 1.5 hr at 37°C. Meanwhile, the barcode DNA was hybridized to capture barcode strands. The capture strands were already added to the chip by a microarrayer. After incubation, slides were immersed for 1 min in a solution of (425 ml Nano(pure)H_2O + 25 ml 20 X SSC + 500 ul 10% SDS), a new hybridization buffer that removes DTT and any unbounded DNA. The chips were spun dry and a new gasket was put on. 16 ul of a universal nanoparticle probe that consists of functionalized DNA was added to each of the four wells. The universal probe had undergone a 1:20 dilution in a buffer solution of (1 X SSC + .05% Tween 20 + 10% formamide). The slides were then incubated at 37° C for 45 min. After incubation, the hybridized universal probe underwent silver staining amplification. Silver staining amplification was accomplished by mixing 1 ml of silver solution A and 1 ml of silver solution B. Silver solution A contains silver ions, and silver solution B contains quinine as a reducing agent, to turn the silver ions into silver metal. This solution was then poured on the slides for 5 min to amplify the signal denoting detection of the ADDLs. This process results in gray spots that can be seen with a Verigene ID or Scanometric system (Nanosphere Inc., Northbrook, Illinois) that measures scattered light.[1] The data were then quantified using a molecular dynamics program called Gene Pix that calculates the spot intensity and subtracts the local background from the developed spot on the slide. (Data are shown in Figures 1a–d and Figure 2.)

Results and Discussion

This study used plasma samples from the Cognitive Neurology and Alzheimer's Disease Center at Northwestern University's Feinberg School of Medicine. Of the 16 samples used, 8 were from known AD patients and 8 from control patients. Results showed that more control patients had a higher average raw intensity of ADDLs than patients with Alzheimer's (shown in Figure 2). The small sample size makes the distinction between AD patients and control patients vague. Other factors could also have been involved. In addition, this could have caused a significant amount of nonspecific binding of ADDLs in the assay. Also, antibodies functionalized with the Au and MMPs used in the assay could have become denatured as well. Problems with the data could also be due to washing protocols, funtionalization of Au and MMPs, the condition of the universal probe, or the freezing of the barcodes for later use in the assay.

Conclusions

Many factors can affect the detection of proteins in the bio-barcode assay. Every step in the assay has to be performed precisely and accurately to obtain reliable results. Also, a large sample size is needed for testing the statistical accuracy of the data. Here, only a small sample size was taken, and a distinction between the AD patients and the control group could not be successfully established. Some modifications must take place before this assay can be used clinically, such as removing unbound Au particles and ensuring the specific binding of the protein of interest. For future work, it should be kept in mind that the assay runs efficiently when the barcodes are not frozen but rather used immediately after they are obtained. This assay's great promise includes its ability to test a greater number of samples than can be tested in current and conventional assays, allowing for the production of more statistical data and the resulting ability to make clear distinctions. Also, in previous reports this assay has been shown to detect proteins at very low concentrations because of its ultra sensitivity. The successful detection of ADDLs in CSF at low concentrations offers great possibilities for future clinical diagnostic applications.

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