Nanoparticle-based detection in cerebral spinal fluid of a soluble pathogenic biomarker for Alzheimer’s disease

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The recently developed ultrasensitive bio-barcode assay was used to measure the concentration of amyloid-β-derived diffusible ligands (ADDLs), a potential soluble pathogenic Alzheimer’s disease (AD) marker, in the cerebrospinal fluid (CSF) of 30 individuals. ADDL concentrations for the subjects diagnosed with AD were consistently higher than the levels in the CSF taken from non-demented age-matched controls. Studies of ADDLs or for any other potential pathogenic AD markers in CSF have not been possible because of their low concentration in CSF (<1 pm). This study is a step toward a diagnostic tool, based on soluble pathogenic markers for the debilitating disease.

Alzheimer’s disease (AD) is the most common neurodegenerative dementia with an average death prognosis of 9 years (1). There is no definitive diagnosis of the disease other than postmortem identification of senile plaques and neurofibrillary tangles in the brain (2, 3). Premortem diagnosis, based on a patient’s clinical history; in vivo brain imaging (4–7); and neuropsychological, cognitive, and neurological tests, is only 85% accurate (8). There are two general approaches for detecting soluble markers for AD. One approach is based on measuring the total tau protein or amyloid-β protein concentration in cerebrospinal fluid (CSF) or plasma (9–12). This approach is hampered by significant overlap of such marker levels in healthy and unhealthy subjects and has led to inconclusive results (13–16). The other approach targets only the suspected pathogenic markers, such as cleaved tau protein, phosphorylated tau protein (17), or amyloid-β-derived diffusible ligands (ADDLs). Although this approach to detecting pathogenic markers might lead to more definitive results, the concentrations of such markers in CSF are so low in the early stages of the disease that they cannot be identified accurately with conventional ELISA or blotting assays. Here, we demonstrate that the ultrasensitive nanoparticle-based bio-barcode assay can be used to determine the approximate ADDL concentration in CSF taken from 30 subjects, 15 of whom were diagnosed with the disease through postmortem analysis of the brain and 15 of whom were found not to have the disease.

Since its invention, the bio-barcode assay has become a powerful analytical tool for the detection of both protein and nucleic acid targets (18, 19) (Scheme 1). In a previous report, we were able to detect prostate-specific antigen with a sensitivity six orders of magnitude greater than the conventional ELISAs for the same target (18). This extraordinary sensitivity opens up the possibility of considering for disease diagnosis biomarkers that could not be used with conventional technology. ADDLs and many of the other potential pathogenic markers for AD provide an excellent test case to evaluate the capabilities of the barcode assay and, in the process, could provide important leads to useful diagnostic systems for studying and ultimately treating the disease (1, 20–22).

Abbreviations: AD, Alzheimer’s disease; ADDL, amyloid-β-derived diffusible ligand; pAb, polyclonal Ab; CSF, cerebrospinal fluid; NP, nanoparticle; MMP, magnetic microparticle.

See Commentary on page 2263.

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TAAGACGAGGCAATCATGCAATCTGAATGCGA\textsubscript{10-20C2 mAb) were generated and characterized as de-
IL). Abs targeting ADDLs in the bio-barcode assay (M90 pAb from the supernatant. The concentration of synthetic ADDLs
with 1 U.K.). The 30-nm particles (1 ml) were initially functionalized
purchased and used as received from BB International (Cardiff,
nanometer diameter) were prepared by following the standard methods
are also available on request.

**CSF Preparation.** Premortem samples (provided by John Lee,
Loyola University Medical School, Maywood, IL) were obtained
via lumbar puncture and kept frozen until used. Postmortem
samples were obtained from the Religious Order Study repos-
tory maintained by Rush University Medical School (Chicago).
The latter samples were obtained postmortem from 10 mild- to
severe-AD-diagnosed subjects and 10 age-matched noncogni-
tively impaired control cases. For the latter 20 subjects, results
of the National Institute on Aging–Reagan Institute and Mini
Mental State Examination (MMSE) neuropsychological tests
are also available on request.

**Antigen Isolation and Ab Expression for ADDLs.** A\textsubscript{B1-42} peptide
(California Peptide Research, Napa, CA) was used to prepare
synthetic ADDLs according to published protocols (23). An
aliquot of A\textsubscript{B1-42} was dissolved in anhydrous DMSO to a
concentration of 22.5 mg/ml (5 mM), pipette-mixed, and further
diluted into ice-cold F12 medium (phenol-red-free) (1:50 dilu-
tion; BioSource International, Camarillo, CA). The mixture was
quickly vortexed, incubated at 6–8°C for 24 h, and centrifuged
at 14,000 \times g for 10 minutes, and the oligomers were collected
from the supernatant. The concentration of synthetic ADDLs
was determined by using a microBCA assay (Pierce, Rockford,
IL). Abs targeting ADDLs in the bio-barcode assay (M90 pAb and 20C2 mAb) were generated and characterized as described (23).

**NP Synthesis and Modification.** Citrate-stabilized Au NP probes (13
nm diameter) were prepared by following the standard methods
given in ref. 28, and 30-nm-diameter Au NP (0.5 nM) were
purchased and used as received from BB International (Cardiff,
U.K.). The 30-nm particles (1 ml) were initially functionalized
with 1 \mu g of pAb antigen-specific Ab (M90) in a basic aqueous
solution (pH 9). The particles were modified subsequently with
thiolated DNA (final concentration, 2 \mu M) by slow salt aging (40
h) to a final concentration of PBS (0.1 M NaCl in 0.01 M of
phosphate buffer, pH 7; denoted as PBS unless indicated oth-
erwise). Unbound thiolated DNA was removed by repetitive
centrifugation (15,700 \times g for 30 min) of the particles, followed
by rinsing and resuspension in PBS. The thiolated DNA is
complementary to the bio-barcode DNA strand that serves as
the amplification target. The barcode DNA was added (final
concentration, 2 \mu M) to the NP solution and allowed to hybrid-
ize at room temperature for 4 h. The NPs were centrifuged at
15,700 \times g, and the supernatant containing excess barcode DNA
was removed. The particles were resuspended in 0.1 M PBS, and
the procedure was repeated three times.

The NPs used in the scanometric assay were prepared according
to the methods published in ref. 28. The concentration of the
NPs was calculated based on extinction spectra by using known
values (28) of the extinction coefficients for gold NPs:

\[
\varepsilon_{520 \text{nm}} = 2.7 \times 10^8 \text{ and } \varepsilon_{520 \text{nm}} = 3.7 \times 10^9 \text{ cm}^{-1}\text{M}^{-1}.
\]

The diameters of the synthesized NPs were determined by
transmission electron microscopy by using an 8100 instrument
(Hitachi, Tokyo).

**Functionalization of MMPs.** The amino-functionalized MMPs (100
\mu l, 50 mg/ml aqueous solution, 1 \mu m diameter polyamine
particles with iron oxide cores, Polysciences) were modified with
100 \mu g of antibodies according to the manufacturer’s protocol.
The Abs were mAbs specific to ADDL (20C2).

**Functionalization of Glass Slides.** Functionalized glass slides were
modified with half-complementary thiolated capture DNA
strands (100 \mu M) using a microarrayer (Affymetrix, Santa Clara,
CA) according to a standard published procedure (18). The
DNA strands were covalently immobilized on the chip, the
unbound strands were washed away with H\textsubscript{2}O, and the residual
binding sites were passivated by immersion in 40 mM mercap-
tosuccinic acid for 30 min, followed by repetitive washing with
H\textsubscript{2}O.

**Bio-Barcode Assay.** In a typical assay, 10 \mu l of CSF or 10-\mu l
aliquots of ADDL at known concentrations ranging from 100 aM
to 100 fM were added to 50 \mu l of MMP solution (5 mg/ml) and
allowed to react under vigorous stirring at 37°C for 1 h. After
magnetically immobilizing the MMPs, the unbound antigens
were removed by repeated washing with PBS. The MMPs and
antigen–target complexes were magnetically separated, and 50
\mu l of 0.1 nM NP probe (Ab-functionalized and DNA-modified)
was added and stirred vigorously at 37°C for 30 min to bind the
target antigen–MMP complex. The sandwich complexes were

![Scheme 1](https://example.com/scheme1.png)

**Scheme 1.** The bio-barcode amplification assay. The assay uses MMPs functionalized with mAbs that recognize and bind ADDLs. The ADDLs are then sandwiched with an NP probe, modified with double-stranded DNA and an anti-ADDL pAb. After repeated washing while using a magnet to immobilize the MMPs, a dehybridization step releases hundreds of barcode DNA strands for each antigen-binding event.
then magnetically separated and washed four times with 100 μl of PBS solution. In the final step, 50 μl of H2O was added and the solutions were stirred vigorously at 60°C for 30 min to allow for full dehybridization of the target DNA. The complexes were again separated magnetically, and the supernatant containing the barcode DNA was collected for quantification by the scanometric detection method (29).

**Scanometric Detection.** Light scattering by the silver-amplified gold particles was quantified with a commercial image analyzer (Verigene ID, Nanosphere, Northbrook, IL) and the scattering image of the whole slide was collected as a .tif file (16-bit resolution, maximum 216 = 65,536 arbitrary units) with a typical acquisition time of 20 ms. The grayscale intensity was quantified by using the commercially available software IMAGEQUANT.

## Results

In a typical assay, tested samples consisted of either known concentrations of synthetic ADDLs in PBS buffer or CSF samples from subjects obtained via lumbar puncture or extraction from the posterior ventricle. A known volume of each sample was mixed with NPs modified with double-stranded oligonucleotides and MMPs, both functionalized with antigen-specific Abs (see Scheme 1). Both MMPs and NPs are present in excess so that the target antigens are captured effectively, thereby overcoming slow binding kinetics. Binding of the Ab-modified particles to the target antigen results in a sandwich complex involving the NPs and magnetic particles. Magnetic separation of the sandwiched complexes results in efficient separation from the rest of the sample. The double-stranded DNA strands on the NPs are then dehybridized, releasing hundreds of barcodes per target protein into the supernatant. The released barcode DNA strands are easily detected by using the scanometric method (29), and the results are recorded (Scheme 2) with a Verigene ID system (Nanosphere, Northbrook, IL).

Before analyzing subject samples, a calibration curve for synthetic ADDL concentration in PBS buffer was established (Fig. 1). The assay is semiquantitative and exhibits an analytical target concentration range of three orders of magnitude, with a lower limit of detection at ~100 aM. At high target concentrations (>500 fM), the assay response becomes nonlinear, and eventually, the signal plateaus. The calibration curve shown in Fig. 1 finally exhibits a plateau at high concentrations (>500 fM) because of the stoichiometry changes from 1:1:1 to 1:>1:1 (MMP/ADDL/NP), whereas at high ADDL concentrations the MMPs bind more than one NP carrying barcode DNA. The resulting calibration curve establishes the analytical range available to the bio-barcode assay for this soluble marker under the stated conditions, that spans from low attomolar to high femtomolar.

CSF samples from thirty subjects were then evaluated with the assay (Fig. 2). We obtained 10 samples by means of premortem lumbar puncture and 20 samples by means of postmortem extraction from the posterior ventricle of the brain. All cases underwent a standard and uniform neuropathological evaluation for the diagnosis of AD using well-established diagnostic criteria, including the Braak and Braak score (30, 31) and the CERAD (Consortium to Establish a Registry for Alzheimer’s Disease) criteria (32). Brain extract from a definitively diagnosed AD patient, in which ADDL concentration is known to be high (>1 pM), served as a positive control yielding a saturated signal, and brain extract obtained from a healthy subject without clinical and pathological signs of AD was used as a negative control. The 30 assayed samples were obtained from subjects whose AD histopathological symptoms ranged from null to severe. The ADDL concentration in the CSF for each patient is estimated by comparison of grayscale intensity in the scanometric assay with the calibration curve in Fig. 1.

## Discussion

The assays for control subjects showed a consistently lower level of ADDLs than the assays for subjects diagnosed with the disease (Fig. 2). The ADDL concentration medians of the two groups are ~200 aM and 1.7 fM, respectively (statistical value from unpaired t test, $P < 0.0001$). The highest ADDL concentration in the control group is <0.5 fM, and most of the ADDL concentrations in the AD group are >0.5 fM. Only two indi-
vividals diagnosed with AD exhibited overlap with the control population of healthy subjects. One subject was an aged male that scored very high on the Mini Mental State Examination (MMSE), which is inconsistent with the later pathological stages of the disease, whereas the other subject had pathological signs of infarctions in addition to AD. These two AD subjects that are outliers in our assay could be false positives of current diagnostic methods that consider both clinical and pathological data.

Interestingly, one nondemented subject (the lowest of control group) had a moderate number of plaques but no ADDLs in CSF. These data are consistent with the idea that soluble oligomers, rather than plaques, may be the Aβ species most relevant to dementia. The presence of ADDLs in CSF may provide a soluble marker to establish AD, and ADDL concentration, in conjunction with the present neuropsychological tests, may act as an accurate marker to delineate the different stages or forms of dementia.

In conclusion, this study is significant for a number of reasons. First, it shows that the ultra-high-sensitivity bio-barcode assay can be used to measure the concentration of the pathogenic ADDL in CSF at clinically relevant concentrations. This advance opens up the opportunity to study a wide range of potential pathogenic markers in CSF, which could lead to very informative diagnostic tools for the disease. Second, it suggests that the soluble pools of ADDLs that exist in the human brain extend to the CSF, and that elevated levels of ADDLs correlate with the presence of the disease. Third, the approach of using pathogenic markers in CSF combined with the barcode assay points toward a potential reliable detection method for diagnosing AD that is faster, higher throughput, and less expensive than current imaging techniques. Before this assay can be used clinically, it needs to be statistically validated with an even larger patient pool.

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