Published in final edited form as:

Integr Biol (Camb). 2011 December ; 3(12): 1188–1196. doi:10.1039/c1ib00018g.

CSF levels of oligomeric alpha-synuclein and beta-amyloid as biomarkers for neurodegenerative disease

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Abstract

Protein misfolding and aggregation is a critically important feature in many devastating neurodegenerative diseases, therefore characterization of the CSF concentration profiles of selected key forms and morphologies of proteins involved in these diseases, including β -amyloid (A β) and α -synuclein (a-syn), can be an effective diagnostic assay for these diseases. CSF levels of tau and A β have been shown to have great promise as biomarkers for Alzheimer's disease. However since the onset and progression of many neurodegenerative diseases have been strongly correlated with the presence of soluble oligomeric aggregates of proteins including various A β and a-syn aggregate species, specific detection and quantification of levels of each of these different toxic protein species in CSF may provide a simple and accurate means to presymptomatically diagnose and distinguish between these diseases. Here we show that the presence of different protein morphologies in human CSF samples can be readily detected using highly selective morphology specific reagents in conjunction with a sensitive electronic biosensor. We further show that these morphology specific reagents can readily distinguish between post-mortem CSF samples from AD, PD and cognitively normal sources. These studies suggest that detection of specific oligomeric aggregate species holds great promise as sensitive biomarkers for neurodegenerative disease.

Introduction

Neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) will affect an increasing number of people as our population ages. For AD alone, over 5 million Americans currently are living with the disease, with nearly half a million new cases expected each year with total yearly economic costs of over \$170 billion.¹ Diagnosis of these diseases is challenging as other neurodegenerative diseases such as Lewy Body Dementia (LBD), frontotemporal dementia and vascular dementia may share similar symptoms, but have different mechanisms and pathology.² Patients in early stages of dementia are often classified as having Mild-Cognitive Impairment (MCI), a term that describes early, nondisabling cognitive disorders.^{3,4} Although MCI describes a transitional state between normal aging and dementia, not all MCI cases progress to dementia.^{4,5}

Pathological changes associated with these different dementias have been shown to occur long before symptoms are evident,⁶ suggesting that an appropriate set of biomarkers would have great promise to study toxic mechanisms and pathways in these different diseases and to facilitate early diagnoses.

Numerous studies have looked at key biomarkers for diagnosing neurodegenerative diseases, especially AD (reviewed in ref. 7) where levels of tau, phosphorylated tau and amyloid-beta 42 (A β 42) have shown promise for predicting AD. A recent study using these biomarkers correctly identified 94% of autopsy verified AD cases and also accurately predicted (100% sensitivity) which MCI cases would progress to AD.⁸ These very promising studies indicate that CSF biomarkers can be a valuable tool to both facilitate diagnosis of neurodegenerative disease and to assess effectiveness of different therapeutic strategies.

While CSF protein biomarkers such as tau and A β hold promise as diagnostics for neurodegenerative diseases, a better more selective diagnostic biomarker set can potentially be obtained by detecting specific toxic protein species that are associated with each disease. Since many neurodegenerative diseases are correlated with misfolding and aggregation of different target proteins; amyloid-beta (A β) with AD, alpha-synuclein (a-syn) with PD, LBD and other synucleinopathies, and tau with AD and various tauopathies, specific detection and quantification of levels of each of these different toxic protein species may provide a means study mechanisms of toxicity and progression in these diseases and to presymptomatically diagnose and distinguish between these diseases. For example, a vast amount of literature implicates A β accumulation and plaque formation as being central to the progression of AD,⁹ however, while the presence of amyloid plaques does not correlate well with the progression of AD,^{10,11} the presence of various different soluble A β species does.^{12,13} Similarly, soluble aggregate forms of tau have been correlated with AD^{14–16} and soluble forms of a-syn have also been correlated with PD.^{17,18}

Since protein misfolding and aggregation are closely associated with many neurodegenerative diseases, determining the CSF concentration profiles of selected key forms and morphologies of proteins involved in these diseases, including A β and a-syn, can facilitate development of an effective diagnostic assay to help study these diseases. Since specific soluble aggregate morphologies of these proteins are likely present at only very low concentrations in CSF, in order to detect and quantify levels of these proteins, highly selective reagents that specifically recognize each of the target species are needed along with a sensitive biosensor system. We have used novel protocols to isolate reagents that bind specific morphologies of target proteins by combining the imaging capabilities of AFM with the binding diversity of phage display antibody technology.^{19–23} These morphology specific reagents are ideal candidates to determine whether specific aggregate species of A β and a-syn can be detected in CSF and whether they have potential as markers to help study and diagnose neurodegenerative diseases. We have also designed and developed a sensitive label-free biosensor technology operating on the principle of electrochemical impedance spectroscopy that is well suited to identify protein biomarkers in clinical samples.^{24–26} The biosensor has several features that are ideally suited for detecting low concentrations of specific protein morphologies in human samples. First it uses a label-free technology so only a single binding event and no modification of target antigen are needed. Second, the nanoscale array includes a porous filter to prevent cells and other large material from blocking the antibody surface and to confine the target antigen in the porous wells. Third, the sensor can determine antigen concentrations over large ranges with detection limits down to low femtomolar or even attomolar levels. We used the NanoMonitor assay (US patent application number: 20070256941) as a clinical diagnostic tool for simultaneous detection of low nano- and picogram/ml levels of various target antigens in patient serum samples.^{24–26} The high sensitivity of the Nanomonitor combined with the high selectivity of

the morphology specific nanobodies provides a uniquely powerful tool to determine whether CSF levels of various different aggregate morphologies of A β and α -syn have potential as biomarkers to study and diagnose neurodegenerative diseases. Here we show that morphology specific nanobodies in conjunction with an electronic impedance biosensor can readily distinguish post-mortem CSF samples taken from human AD, PD and age matched non-diseased sources.

Materials and methods

In this section, an overview of the biosensor design and operation is given, followed by the experimental methods utilized.

Fabrication of the biosensor device

The biosensor device is comprised of three integrated parts; (a) a printed circuit board platform, (b) a nanoporous alumina membrane and (c) a silicone micro fluidic chamber. The measurement surface for the biosensor device is a printed circuit board platform comprised of inter-digitated working and counter electrodes. The tin oxide electrodes are 800 μ m in width, 5mm in length and 800 nm in thickness with rounded edges to minimize fringe effects during the application of a sinusoidal voltage input signal (Fig. 1A).

A nanoporous alumina membrane is soldered onto the inter-digitated electrodes generating a high density array of nanowells. The membrane is 250 nm thick, has a lateral diameter of 13 mm with pore diameters of 200 nm. The porosity of the membranes varies between 25% and 50%. An alumina membrane was utilized since it offers electrical isolation between each individual nanowell, as well as good biocompatibility (Fig. 1B).

Finally, a circular silicone chamber encloses the nanotextured electrode surface to confine the fluid onto the device surface and prevent evaporation which could lead to electrical signal instability. The chamber has a maximum working volume of 1.6 ml. Thus, the combination of the alumina membrane on a PCB substrate enclosed by a silicone chamber forms an inexpensive bio-sensing device capable of detecting various bio-molecules (Fig. 1C).

Detection methodology – electrochemical impedance spectroscopy (EIS)

The electronic biosensor measures impedance changes to the electrical double layer at the solid–liquid interface within the nanowells induced when target proteins contained in the sample bind to reagents such as antibodies immobilized on the sensor surface. Impedance measurements provide very detailed information about the electrical changes occurring at the conductive or semi-conductive interfaces.

When target antigens bind immobilized antibody inside the nanowells, the double layer capacitance changes due to the change in the surface charge concentrations. Thus, the double layer capacitance directly correlates to the amount of binding taking place at the solid–liquid interface, and the amount of binding is directly proportional to the concentration of the target species. Thus, by characterizing the double layer capacitance, we can get an accurate estimate of the concentration of the target species. The changes to the double layer capacitance can be represented as the measured impedance changes especially at low frequencies (*i.e.* below 1 kHz). In the sensor configuration used here, redox probes are not used, and it can be assumed that all the conduction occurring at the interface is non-faradaic in nature, so the charge distribution dynamics at the metal–solution interface characterizing the bio-molecular interactions at the surface can be modeled using the Helmholtz–Gouy–Chapman model with Sterns correction.^{27,28} Since binding of antigens to the nanobodies is free of any bio-chemical mediators, the impedance changes within the electrical double layer

(Fig. 1D) is non-Faradaic, and the electrical circuit model of the sensor can be represented as a simple resistive-capacitive (RC) series circuit whose values are extracted by a frequency response analyzer potentiostat.²⁴ For probing the impedance changes to the electrical double layer of the nanowell electrodes, a very small amplitude sinusoidal voltage is applied to the electrochemical system, and the output current response is sensed, the ratio of the applied voltage phasor to the output current phasor is the resulting impedance, which is characterized using a frequency response analyzer (Gamry Industries Reference 600 potentiostat).

Another commonly used electrochemical immuno-sensing technique is the pulsed amperometry. This method involves the immobilization of an immune-reagent component on the electrode transducer and the use of an electrochemical active substance produced by enzymatic reaction for signal generation.⁶⁷ As simple as this appears, there can be numerous problems associated with an inadequate supply of enzyme inhibitors in the sample, instability of the enzyme over time, irreproducibility of the electrode kinetics for reoxidizing reagent or reducing oxidising agent, redox active interferences which either react at the electrode and/or couple with the reagent couple, and inadequate temperature control.⁶⁸ The EIS technique eliminates most of these problems since it doesn't rely on the redox properties of the analyte and doesn't need an enzyme inhibitor. Another fundamental difference between the two techniques is the sensing mechanism. Amperometry involves detection of ions in the solution by applying a fixed voltage through electrodes, and measuring the current/change in current; whereas EIS involves characterizing the electrical double layer at the electrode by sweeping a range of frequencies, and measuring the current.

Nanobody immobilization to sensor surface

Nanobodies are immobilized onto the electrode sensor surface using a chemical linker. The electrode surface is first amine functionalized using 3-Aminopropyl Triethoxysilane (APTES, 2% in acetone buffer, Thermo Scientific Inc.). A 100 μ l aliquot of 2% APTES is applied on the electrode surface and incubated at room temperature for 30 s. Excess APTES was then removed by flowing acetone over the surface. The alumina membrane is then soldered to the silanized electrode surface. Then 3,3'-dithiobis succinimidyl propionate (DSP, Thermo Scientific Inc.) dissolved in DMSO solvent (4 mg ml⁻¹) is used to cross link the nanobodies to the electrode surface. The DSP (thiol linker) is added for 30 min to allow conjugation to the silanized electrode surfaces which form the base of the nanowells. After conjugation of the linker to the nanowell surfaces (primarily to the base of the nanowells), a 40 μ l aliquot of the nanobody (1 mg ml⁻¹) is added for 15 min, followed by addition of 40 μ l of Bovine Serum Albumin (BSA) (2 mg ml⁻¹) to block any unbound amine sites on the sensor surface. Phosphate buffered saline (1xPBS) is used to prepare antibody aliquots and for the wash steps due to its isotonic properties.

Preparation of CSF samples

Post mortem CSF Samples from AD, PD, MSA and non-diseased autopsy confirmed sources (Table 1) were generously provided by Dr Thomas Beach (Civin Laboratory for Neuropathology, Banner/Sun Health Research Institute, Sun City, AZ). The post-mortem interval for collection of samples is less than three hours. Samples were diluted to one-tenth of their original concentration in 1X PBS buffer and frozen.

Expression and purification of nanobodies

D10, D5 and A4 nanobodies were produced and purified essentially as previously described.^{21,23} D10 was used to calibrate the sensor chip since it binds to all forms of a-syn including monomeric³⁰ facilitating preparation of a-syn standards of known concentration. The D5 nanobody specifically recognizes a small oligomeric a-syn species,²² while A4

specifically recognizes a small oligomeric A β species.²¹ Nanobody specificity was demonstrated by several different assays including time course dot blot, ELISA, western blot and Atomic force microscopy based assays.^{21,22,31} The supernatant and cell lysate from a 1L culture were combined and concentrated in a tangential flow filter (Millipore) using a 10 kDa filter membrane (Millipore). Concentrated samples were purified using a protein A-Sepharose column (GE healthcare, NJ) as previously described.²¹ Fractions containing nanobody were pooled, dialyzed against PBS, lyophilized and stored at -20°C . The purity of the nanobody sample was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide gels (Bio-Rad, Hercules, CA) and western blotting, and the concentration was determined using bicinchonic acid (BCA) protein assay (Pierce, IL).

Sensor calibration

A-syn was prepared and purified in our lab as previously described.^{22,29} Purified stocks of a-syn were lyophilized and stored at -80°C . Stocks were first dissolved in DI water and subsequent dilutions were made in Tris buffer (25 mM Tris, 150 mM NaCl, pH 7.4). A 40 μl aliquot of test sample is added, incubated for 10 min, and then impedance measurements are taken.

Results

Operating parameters and detectability limits of biosensor with nanobodies as capturing agents

Specific aggregate morphologies of a-syn and A β are likely to be present in CSF samples only at very low concentrations (nanomolar or less), therefore successful detection of these targets requires a biosensor with very low detection limits. In order to determine the sensitivity of the biosensor, we utilized the D10 nanobody as a capture agent. D10 recognizes all forms of a-syn,³⁰ and therefore we can accurately control target a-syn concentrations. The first step to determine biosensor sensitivity is to determine suitable electrical parameters which will enable detection of bound target using electrochemical impedance spectroscopy.

Since binding of antigen to target takes place over a high density array of nano-pores, the impedance signal we obtain correlates to the average signal obtained over all the pores, ensuring that even if some pores do not contain immobilized capture agent the measured impedance will be reproducible within an acceptable margin of error. The dimensions of the electrical double layer within the nanopores is approximately 50 nm. Based on previous studies,^{24,25} a 100 mV peak to peak pulse should be utilized to characterize changes to the capacitance of the electrical double layer induced by biomolecule binding. The second parameter that needs to be defined is the sensing frequency. Since double layer capacitance dominates the impedance spectrum for frequencies less than 1 KHz in order to determine the optimum frequency for these studies, we tested the frequency response by adding a range of monomeric a-syn concentrations (1 ng ml^{-1} to $1\text{ }\mu\text{g/ml}$) to immobilized D10 using a frequency range from 50 Hz to 1MHz. A frequency of 100 Hz gave maximum visible shifts in the impedance induced by biomolecule binding (Fig. 2).

Using the defined electrical parameters, we generated a calibration curve using different concentrations of a-syn using two controls; BSA was added to immobilized D10 to test for nonspecific binding to nanobody, and a-syn without immobilized D10 was used to measure background a-syn binding to the sensor surface.

From the calibration curve, we can accurately detect antigen down to a limit of detection (LOD) of $1\text{ picogram ml}^{-1}$ (Fig. 3) indicating that it should be possible to detect low

femtomolar concentrations of target antigen in clinical patient samples. The results also indicate that the target antigen, α -syn, binds to the immobilized nanobody and not the conjugation linker since the signal obtained with antigen binding to immobilized nanobody, even at antigen concentrations six orders of magnitude lower, is higher than the signal observed without nanobody (Fig. 3). The nanobody also specifically reacts with the α -syn target since no signal is observed with the control protein antigen, BSA (Fig. 3).

After demonstrating that we can detect femtomolar concentrations of α -syn target using immobilized nanobodies, we next determined whether we could detect specific morphologies of α -syn in post-mortem CSF samples. While we utilized the D10 nanobody, which recognizes all forms of α -syn, for the calibration studies since we could accurately measure the concentration of monomeric α -syn present, in order to detect specific oligomeric forms of α -syn and $A\beta$ in CSF samples, we next utilized two other nanobodies which selectively recognize either a specific oligomeric form of α -syn (D5) or of $A\beta$ (A4). We immobilized either D5 or A4 to sensor chips and then analyzed nine different CSF samples, three taken from 3 AD patients, 3 PD-like (2 PD and one Multiple System Atrophy (MSA) which shares some similar symptoms to PD), and 3 aged matched non-demented control samples (ND). Since all nine samples could not be sequentially analyzed on a single chip, we divided the samples into groups of three, where each group contained one ND, one AD and one PD-like sample.

The experiments were repeated several times and performed simultaneously utilizing multiple sensor chips. In order to control for inter-sensor variability associated with the sensor manufacturing process where there is heterogeneous integration of multiple materials, we utilized a common sample in each data set (AD1) performed on each chip, and normalized data from different chips to this common reference point. An illustrative example of the comparison of percentage change in impedance values obtained in the raw data compared to normalized data is shown in Table 2. The percentage change in impedance values after normalization to a common reference point are shown for all nine CSF samples obtained with immobilized D5 (Fig. 4A) and A4 (Fig. 4B).

Oligomeric species of both α -syn and $A\beta$ are readily detected in post-mortem CSF samples even when assayed at 10-fold dilution (Fig. 4A and B). The results indicate that the levels of oligomeric α -syn and $A\beta$ species in CSF varies depending on the disease, and that these oligomeric species have great promise as biomarkers for distinguishing between different neurodegenerative diseases.

Discussion

CSF levels of $A\beta$, tau and phosphorylated tau were shown to have promise as biomarkers for diagnosing AD.^{7,8} However increasing evidence indicate that various soluble aggregated oligomeric forms of $A\beta$, α -syn and tau are the relevant toxic species in different neurodegenerative diseases, and specific detection of different aggregate species in CSF may provide a more refined and powerful tool to facilitate early and accurate diagnosis of a variety of neurodegenerative diseases and to study the mechanisms involved in the onset and progression of these diseases. Protein aggregation is a common thread behind numerous neurodegenerative diseases including AD, PD, LBD, tauopathies and synucleinopathies. Aggregation of $A\beta$ has been correlated with AD, aggregation of α -syn with PD, LBD and other synucleinopathies, and aggregation of tau with AD and various tauopathies. While the presence of fibrillar aggregates of these different proteins has been a classic diagnostic feature of the respective diseases, increasing evidence suggests that soluble oligomeric forms of these proteins are the relevant toxic species. During the polymerization process from monomeric to fibrillar form, each of the protein species must pass through different

oligomeric states, suggesting that various oligomeric species may represent earlier biomarkers for these diseases compared to the presence of fibrillar forms.

A rapidly growing body of evidence indicates that oligomeric forms of A β are key factors in the onset and progression of AD. A β forms a number of soluble intermediate or metastable structures which may contribute to toxicity (reviewed in ref. 32 and 33). Cortical levels of soluble A β correlate well with the cognitive impairment and loss of synaptic function.^{12,13} Small, soluble spherical or annular aggregates of A β were shown to be neurotoxic,^{34–36} and oligomeric forms of A β , created *in vitro* or derived from cell cultures inhibit long term potentiation (LTP).^{35,37,38} The concentration of oligomeric forms of A β are elevated in transgenic mouse models of AD³⁹ and human AD brain⁴⁰ and CSF samples⁴¹ and the presence of an SDS stable dimeric form of A β associates well with dementia in AD patients.⁴² Disruption of neural connections near A β plaques was also attributed to oligomeric A β species,⁴³ a halo of oligomeric A β surrounds A β plaques causing synapse loss,⁴⁴ and oligomeric A β was shown to disrupt cognitive function in transgenic animal models of AD.^{45–47} Different size oligomers of A β have been correlated with AD, including a 56 kD aggregate⁴⁸ and smaller dimeric, trimeric and tetrameric species.^{32,38,49,50} Therefore specific detection of soluble oligomeric A β species holds great promise as a biomarker for studying the progression of AD.

Similarly, formation of oligomeric aggregates of a-syn has also been correlated with PD and other synucleinopathies. A-syn is a major component of Lewy bodies and neurites.^{51,52} While wild-type and mutant forms of a-syn associated with familial cases of PD, A30P, E46K and A53T, can assemble into Lewy body like fibrils *in vitro*,^{53–57} the mutations increase the total rate of oligomerization compared to the wild-type form of a-syn.^{53,55,58} Different morphologies of a-syn have different affinities for various membranes, and both oligomeric and fibrillar forms^{59–62} have been shown to disrupt membrane permeability and integrity. Aggregated forms of a-syn were shown to induce toxicity in dopaminergic neurons *in vivo*⁶³ and several different oligomeric morphologies were shown to each have different toxic mechanisms and effects on cells.⁶⁴ Oligomeric forms of a-syn were shown to be toxic to neuronal cells,²² and toxic oligomeric a-syn forms were identified in living cells,⁶⁵ in human plasma from PD patients,¹⁸ and in human PD brain tissue.^{22,23} Elevated levels of soluble oligomeric a-syn species were also detected in post-mortem brain extracts from patients with LBD,¹⁷ even though monomeric a-syn levels in CSF were not able to discriminate between LBD and AD.⁶⁶ Therefore the presence of various oligomeric a-syn species in CSF is also a very promising biomarker for studying the progression of various neurodegenerative diseases.

We developed a novel biosensor for sensitive detection of biomolecules from clinical samples^{24–26} and show here that the nanomonitor has sufficient sensitivity to detect low femtomolar concentrations of target directly from human CSF samples using single chain antibody fragments or nanobodies as the capture agent. We also developed technology which enables us to isolate nanobodies that selectively recognize specific protein morphologies,¹⁹ and have isolated nanobodies that bind two different oligomeric a-syn species,^{22,23} and two different oligomeric A β species.^{20,21} The different oligomer specific nanobodies do not show cross-reactivity, so the nanobodies binding oligomeric A β do not bind oligomeric a-syn and *vice versa*. The different aggregate species recognized by each of the morphology specific nanobodies can be detected in post-mortem human AD or PD tissue, and can distinguish between AD, PD and healthy brain tissue.^{20–23} Therefore these nanobodies represent excellent tools to detect specific oligomeric aggregates of A β and a-syn in both clinical and animal model samples.

Here we show that detection of an oligomeric A β species in CSF samples using the A4 nanobody very sensitively distinguishes the AD CSF samples from the PD and ND samples (Fig. 4B). The oligomeric a-syn species recognized by the D5 nanobody, is present in highest concentrations in the PD samples, lower concentrations in the AD samples and lowest concentrations in the ND and MSA samples (Fig. 4A). Interestingly, the a-syn species recognized by D5 readily distinguishes between the PD and MSA CSF samples indicating the value of this technology in studying disease mechanisms. Using the results obtained with both the A4 and D5 nanobodies, we can clearly distinguish between AD, PD, and ND or MSA samples, where ND cases do not show above threshold levels of target with either nanobody, PD cases are distinguished by positive binding signal with D5, but not A4, and AD cases show a positive signal with A4 and also D5 in some cases (Fig. 4C). In order to determine whether detection of specific oligomeric forms of proteins are useful as biomarkers for studying and diagnosing neurodegenerative disease, a much larger CSF sample size will need to be analyzed using the various different morphology specific nanobodies against a-syn^{22,23} and A β .^{20,21}

Conclusion

Diagnosis of neurodegenerative diseases including Alzheimer's, Dementia with Lewy Bodies, frontotemporal dementia and Parkinson's diseases is a challenging prospect. Since misfolding and aggregation of proteins including A β , a-syn and tau have been correlated with these neurodegenerative diseases, the presence of different forms of these target proteins in clinical samples has great promise as potential biomarkers to both study and diagnose these diseases. CSF levels of different A β and tau species can correctly predict progression to AD validating this concept. Since increasing evidence indicates that small soluble oligomeric forms of proteins including A β and a-syn are the relevant toxic species associated with neurodegeneration, oligomeric forms of these proteins may be sensitive biomarkers to study the onset and progression of these different neurodegenerative diseases. Here we show that the presence of different protein morphologies in human CSF samples can be readily detected using highly selective morphology specific reagents in conjunction with a sensitive electronic biosensor. We further show that using morphology specific reagents, we can readily distinguish between post-mortem CSF samples from AD, PD and cognitively normal sources. These studies suggest that detection of specific oligomeric aggregate species holds great promise for use as sensitive biomarkers for neurodegenerative disease.

Acknowledgments

This work was supported by grants from the Arizona Biomedical Research Commission, and the Arizona Department of Health Services for the Arizona Alzheimer's Consortium, the Michael J. Fox Foundation and Office of Naval Research under grant number N00014-07-1-0457. We are grateful to the Banner/Sun Health Research Institute Brain Donation Program of Sun City, Arizona for the provision of human brain tissue. The Brain Donation Program is supported by the National Institute on Aging (P30 AG19610 Arizona Alzheimer's Disease Core Center), the Arizona Department of Health Services (contract 211002, Arizona Alzheimer's Research Center), the Arizona Biomedical Research Commission (contracts 4001, 0011, 05-901 and 1001 to the Arizona Parkinson's Disease Consortium) and the Prescott Family Initiative of the Michael J. Fox Foundation for Parkinson's Research.

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Insight, innovation, integration

Misfolded and aggregated protein variants contribute to many human diseases including Alzheimer's and Parkinson's diseases. Detection of small toxic oligomeric aggregates of proteins such as beta-amyloid or alpha-synuclein in human cerebral spinal fluid (CSF) or serum samples may provide a means to study disease mechanisms and progression and a means to presymptomatically diagnose these diseases. To detect the presence of different protein variants in CSF, we coupled the use of novel reagents that selectively recognize specific protein isoforms with a novel electronic biosensor that has femtomolar sensitivity and is compatible with untreated human samples. We show that the presence of specific oligomeric protein species in post-mortem human CSF samples can readily distinguish between different neurodegenerative diseases, and this technology has potential to facilitate early diagnosis of these diseases.

Fig. 1.
 (A) Physical layout of the sensor platform on PCB surface (B) Integration of the alumina membrane (C) Incorporation of silicone chamber and (D) Changes to the electrical double layer within each nanowell due to the binding of the target antigens onto the nanobodies.

Fig. 2. Impedance spectrum variation over the frequency range from 50 Hz to 1MHz. The inset shows a zoomed spectrum for various doses at relevant frequencies. 100 Hz showed maximum separation between the spectrums for individual doses, hence, 100 Hz was chosen as the sensing frequency.

Fig. 3. Calibration run of alpha-synuclein in pure samples. The plot also establishes the specificity of the biosensor in identifying the specific interaction between ScFv and alpha-synuclein.

Fig. 4.
(A) Normalized data with D5 antibody (B) Normalized data with A4 antibody (C)
Qualitative representation of the data.

Table 1

Patient CSF sample data sheet giving PMI and diagnosis information

Sample ID	Race (1 = white)	Gender (M/F)	Expired age	PMI	DX summary
ND1	1	M	71	3	Control brain showing only very mild normal aging changes
ND2	1	M	80	2.4	Control brain showing only mild, normal age-related changes
ND3	1	M	65	3.5	Brain within normal limits for age (control brain); Alzheimer type II astrocytosis
AD1	1	F	82	4	Alzheimer's disease (AD); microscopic foci (2), cerebellar cortical sclerosis
AD2	1	F	57	2.16	Alzheimer's disease, presenilin 1 gene mutation (AD); Cerebral white matter rarefaction
AD3	1	F	84	1.83	Alzheimer's disease (AD); Cerebral white matter rarefaction; Eilat crible, basal ganglia; Old infarct, right cerebellar hemisphere
PD1	1	M	80	2	Parkinson's disease (PD); non-diagnostic Alzheimer's changes
PD2	1	M	70	1.83	Parkinson Disease (PD); Dementia (history)
MSA	1	M	83	2.16	Parkinson's disease (PD); dementia (history); Shy-Drager syndrome (autonomic failure)

Generously provided by Dr Thomas Beach (Civin Laboratory for Neuropathology, Banner/Sun Health Research Institute, Sun City, AZ).

Table 2

Percentage change in impedance from baseline for the D5 antibody. The normalized data set represents the raw data set normalized to group 1 AD reference point

	Raw data set			Normalized data set		
	ND	AD	PD	ND	AD	PD
Group 1	4.787248	6.108411	7.383063	4.787248	6.108411	7.383063
Group 2	9.754458	15.3544	16.84676	3.815685	6.006235	6.590008
Group 3	5.5921	7.9426	6.1691	2.871514	4.078518	3.16783

Note: All numbers are percentage change (%).