MORPHOLOGY AND PROTEIN SPECIFIC REAGENTS AS DIAGNOSTICS FOR NEURODEGENERATIVE DISEASES

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ABSTRACT

The invention relates to devices and diagnostic methods using the devices for detecting the presence of neurodegenerative diseases. The device (a “biosensor”) distinguishes between different neurodegenerative diseases and facilitates pre-symptomatic diagnoses.
Figure 1: (A) Nanomonitor (NM) device, (B) Sensing Site – Working (WE) and Counter (CE) electrodes. (C) Input/Output pads connected to sensing site through interconnects, (D) Nanoporous membrane overlying on NM, (E) SEM of membrane, and, (F)
FIGURE 4

Brain derived oligomers

1D synthetic oligomers

3D Synthetic oligomers

C6

E1

A4

FIGURE 5

% impedance normalized to baseline

% impedance change (%)

ND  AD  PD
Figure 6A and 6B

A) % impedance change data normalised to baseline for D5 antibody.

B) % impedance change data normalised to baseline for A4 antibody.
FIGURE 7
Protein

>10H (SEQ ID NO:5)
MAEVQLESGGGLVQPGGSLRSAASGFTSFSSYAMSWVRQAPGKLEGWVSN1SSAQGKLEGWVSSIDSGCASTTYAD
SVKGRFTISRSONSKNTLYQMSLRAEDTVYYCAKASAFDYWQQGTLVTVS6GGGGS7GGGSGGG7GGGSDIQ7MTQS
FSLSA5VGDRVTITCRASQGTLSSYLLNMQKPKFKAPKKIIYASSLSQGSQPSFRSFQGSGTDFTILISSQPEDFA
TYCQSSASPF7FCQG7KVEIKRAAHHHHHGAAEQLISEELNGAA*

>6E (SEQ ID NO:6)
MAEVQLESGGGLVQPGGSLRSAASGFTSFSSYAMSWVRQAPGKLEGWVSY1ASSGGDTTNYADSVKGRTISRDNS
KNTLYQMSLRAEDTVYYCAKASAFDYWQQGTLVTVS6GGGGS7GGGSGGG7GGGSDIQ7MTQSFSLSA5VGDRVT
ITCRASQGTLSSYLLNMQKPKFKAPKKIIYASSLSQGSQPSFRSFQGSGTDFTILISSQPEDFA7TYCQSSASPF7FCQG7KVEIKRAAHHHHHGAAEQLISEELNGAA*

>3Q (SEQ ID NO:4)
MAEVQLESGGGLVQPGGSLRSAASGFTSFSSYAMSWVRQAPGKLEGWVSIQQGKGGQYADSVKGRTISRDNS
KNTLYQMSLRAEDTVYYCAKASAFDYWQQGTLVTVS6GGGGS7GGGSGGG7GGGSDIQ7MTQSFSLSA5VGDRVT
ITCRASQGTLSSYLLNMQKPKFKAPKKIIYASSLSQGSQPSFRSFQGSGTDFTILISSQPEDFA7TYCQSSASPF7FCQG7KVEIKRAAHHHHHGAAEQLISEELNGAA*

>A4 (SEQ ID NO:2)
MAEVQLESGGGLVQPGGSLRSAASGFTSFSSYAMSWVRQAPGKLEGWVA1IQQHTGAPTITYADSVKGRTISRDNS
KNTLYQMSLRAEDTVYYCAKASAFDYWQQGTLVTVS6GGGGS7GGGSGGG7GGGSDIQ7MTQSFSLSA5VGDRVT
ITCRASQGTLSSYLLNMQKPKFKAPKKIIYASSLSQGSQPSFRSFQGSGTDFTILISSQPEDFA7TYCQSSASPF7FCQG7KVEIKRAAHHHHHGAAEQLISEELNGAA*

>E1 (SEQ ID NO:3)
MAEVQLESGGGLVQPGGSLRSAASGFTSFSSYAMSWVRQAPGKLEGWVSSIQPEGRTTAYDSVKGRTISRDNS
KNTLYQMSLRAEDTVYYCAKASAFDYWQQGTLVTVS6GGGGS7GGGSGGG7GGGSDIQ7MTQSFSLSA5VGDRVT
ITCRASQGTLSSYLLNMQKPKFKAPKKIIYASSLSQGSQPSFRSFQGSGTDFTILISSQPEDFA7TYCQSSASPF7FCQG7KVEIKRAAHHHHHGAAEQLISEELNGAA*

>D10L2 (SEQ ID NO:7)
MAEVQLESGGGLVQPGGSLRSAASGFTSFSGMVHWRQAPGKLEGWWASYDQ5KXYADSVKGRTISRDNS
KNTLYQMSLRAEDTVYYCAKASAFDYWQQGTLVTVS6GGGGS7GGGSGGG7GGGSDIQ7MTQSFSLSA5VGDRVTITCR
ASQGTLSSYLLNMQKPKFKAPKKIIYASSLSQGSQPSFRSFQGSGTDFTILISSQGFDIATYCQSSYP7TCPQG7
KVIEKAAHHHHHGAAEQLISEELNGAA*

>G6T (SEQ ID NO:15)
MAEQVLESGGGLVQPGGSLRSAASGFTSFSSYAMSWVRQAPGKLEGWVSA1SGGGTYYADSVKGRTISRDNS
KNTLYQMSLRAEDTVYYCAKASAFDYWQQGTLVTVS6GGGGS7GGGSGGG7GGGSDIQ7MTQSFSLSA5VGDRVTITCR
ASQGTLSSYLLNMQKPKFKAPKKIIYASSLSQGSQPSFRSFQGSGTDFTILISSQGFDIATYCQSSYP7TCPQG7
KVIEKAAHHHHHGAAEQLISEELNGAA*
Figures 9A-9D

9D

Figure 10

![Diagram of a device with labeled components and a graph showing frequency response with various concentrations of alpha synuclein.](image)
Figure 11

Figure 12A-12C
B) % impedance change data normalised to baseline for A4 antibody.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>ND1</th>
<th>ND2</th>
<th>ND3</th>
<th>PD1</th>
<th>PD2</th>
<th>MSA</th>
<th>AD1</th>
<th>AD2</th>
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<td>DS (5% threshold)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A4 (2% threshold)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</table>
MORPHOLOGY AND PROTEIN SPECIFIC REAGENTS AS DIAGNOSTICS FOR NEURODEGENERATIVE DISEASES

RELATED APPLICATIONS


U.S. GOVERNMENT RIGHTS

[0002] This work was supported by the National Institutes of Health Grant R01 AG017984. The United States Government has certain rights to this invention.

BACKGROUND OF THE INVENTION

[0003] As our population ages, neurodegenerative diseases such as Alzheimer’s Disease (AD) and Parkinson’s Disease (PD) will affect an increasing number of people at rapidly increasing cost. For AD alone, over 5 million Americans are living with the disease, with nearly half a million new cases expected next year with total yearly economic costs of nearly $150 billion. Despite many impressive advances in brain research, current clinical diagnostic methods cannot predict the development of AD in cognitively unimpaired elderly individuals. Similar difficulties exist in the diagnosis of other neurodegenerative diseases including PD, Dementia with Lewy Bodies (DLB) and other synucleinopathies and tauopathies, particularly for pre-symptomatic diagnoses.

[0004] Therapies administered very early in the disease course, before clinical signs and advanced brain destruction occur, are more likely to be effective than later treatment, but this is not possible at the present time due to the lack of an adequate predictive test. Any such predictive test must be able to distinguish the different neurodegenerative diseases from normal decline in elderly individuals and from other less common diseases that are clinically similar. Therefore, there is a crucial need for new diagnostic tests that will detect these diseases before they cause disability. While existing clinical diagnostic methods may have a high sensitivity, they generally have much lower specificity [1].

[0005] Certain neurodegenerative diseases correlate with various misfolded and aggregated forms of different target proteins, such as amyloid-beta (Aβ) with AD, alpha-synuclein (α-syn) with PD, DLB and other synucleinopathies, and tau with AD and various tauopathies. Therefore, the concentration profile of various aggregate forms of each of these respective proteins can provide a pre-symptomatic indicator for each disease to facilitate diagnosis and to monitor treatment.

[0006] A vast amount of literature implicates Aβ accumulation as being central to the progression of AD, leading to formation of the Aβ hypothesis [2]. The major weakness of the Aβ hypothesis however, is that the presence of amyloid plaques does not correlate well with the progression of AD [3, 4]. In addition to fibrillar amyloid plaques, Aβ can also form a number of soluble intermediate or metastable structures which may contribute to toxicity (reviewed in [5, 6]). Cortical levels of soluble Aβ correlated well with the cognitive impairment and loss of synaptic function [7, 8]. Small, soluble aggregates of Aβ termed Aβ-derived diffusible ligands (AD-DLs) and spherical or annular aggregates termed protofibrils are neurotoxic [9-11]. Oligomeric forms of Aβ, created in vitro or derived from cell cultures, inhibit long term potentiation (LTP) [10, 12, 13]. These small oligomers are also called “low-n oligomers” (i.e., dimers, trimers, or tetramers). The concentration of oligomeric forms of Aβ are also elevated in transgenic mouse models of AD [14] and in human AD brain [15] and CSF samples [16]. Disruption of neural connections near Aβ plaques was also attributed to oligomeric Aβ species [17]. A halo of oligomeric Aβ surrounds Aβ plaques causing synaptic loss [18], and oligomeric Aβ was shown to disrupt cognitive function in transgenic animal models of AD [19-21]. Different size oligomers of Aβ have been correlated with AD, including a 56 kDa aggregate [22] and smaller trimeric and tetrameric species [5, 23].

[0007] The second major pathological feature of AD brains is the presence of neurofibrillary tangles that contain aggregates of the microtubule associated protein, tau. Tau is a natively unfolded protein, and can aberrantly fold into various aggregate morphologies including β-sheet containing fibrillar forms [24-27]. Tau has 21 different phosphorylation sites [28], and excess phosphorylation can interfere with microtubule assembly. Total tau concentration in CSF has been correlated with AD [29] as has the presence of various phosphorylated tau forms or the ratio of phosphorylated tau to Aβ42 (reviewed in [30]). Levels of oligomeric tau have also been implicated as a potential early diagnostic for AD [31]. Determination of total tau, phosphorylated tau and oligomeric tau concentrations all have potential value as diagnostics for AD.

[0008] Aggregation of alpha-synuclein (α-syn) plays a critical role in PD and synucleinopathies. A-syn is a major component of Lewy bodies and neurites [32, 33]. Wild-type α-syn along with the three mutant forms, A30P, E46K and A53T can assemble into Lewy body like fibrils in vitro [34-38]. Since all of the mutations increase the total rate of oligomerization compared to the wild-type form of α-syn [34, 36, 39], it has been postulated that the intermediate oligomeric morphologies of α-syn are the toxic structures in PD rather than fibrils. A partially folded intermediate of α-syn helps to promote fibril formation in vitro [40] and a protofibrillar form of α-syn is stabilized by formation of a dopamine adduct complex, suggesting a possible connection between this morphology of α-syn and dopaminergic cell death [41]. The different morphologies of α-syn also have different affinities for various membranes, and both the oligomeric forms [42-45] and fibrillar forms [45] have been shown to disrupt membrane permeability and integrity.

[0009] Aggregated forms of α-syn were shown to induce toxicity in dopaminergic neurons in vivo [46] and several different oligomeric morphologies were shown to each have different toxic mechanisms and effects on cells [47]. It has been shown that oligomeric but not fibrillar forms of α-syn are toxic to neuronal cells [48]. Toxic oligomeric α-syn forms were identified in living cells [49], in human plasma from PD patients [50], and in human PD brain tissue[48, 51].

[0010] Clearly protein misfolding and aggregation is a critically important in many devastating neurodegenerative diseases. Therefore, determining how concentration profiles of selected key forms and morphologies of Aβ, tau and α-syn vary in healthy, early, and late stage AD, PD and DLB patients will facilitate development of an effective diagnostic assay for these diseases. In order to define the role of these intermediates in the various diseases, highly specific reagents to
identify the different protein forms are needed. Our lab has developed unique technology that enables us to isolate reagents that bind specific morphologies of a target protein. The inventors have combined the imaging capabilities of AFM with the binding diversity of phage display antibody technology to allow us to identify the presence of specific protein morphologies and then isolate reagents that bind a target morphology [52]. In order to determine how different protein morphologies contribute to different diseases, it is important to understand how the different diseases are currently diagnosed.

The diagnostic term “mild cognitive impairment” (MCI) was originally introduced to define a progressive monosymptomatic amnestic syndrome [53, 54], but more recently has evolved by consensus into an entire classification scheme for early, non-dissolving cognitive disorders [55, 56] that include amnestic MCI (single domain), amnestic MCI-multiple domains, non-amnestic, MCI-single domain, and non-amnestic MCI-multiple domains. The diagnostic criteria for probable AD [57] include dementia with cognitive deficits in at least two cognitive domains including progressive memory loss, normal level of consciousness, onset between ages 40 and 90 years, and without another plausible medical explanation. Diagnostic evaluation of symptomatic patients with MCI and AD include MRI or CT of the brain, neuropsychological testing, and a battery of blood tests all with the intention of excluding potentially reversible (or other) causes of memory loss and dementia. In patients with AD, CSF beta amyloid (Aβ) levels fall and tau levels rise with disease progression, but again are diagnostically equivocal in early stage disease [58]. Blood levels of amyloid have not been shown to be useful diagnostically, at any stage. In asymptomatic people at genetic risk for AD, subtle deficits can be disclosed suggesting that the disease process begins much earlier than symptomatic onset of memory loss. For example, otherwise healthy APOE ε4 homozygotes perform less well on memory tests when they are tired [59], less well on problem solving tasks when they are anxious [60], and both cross sectional [61, 62] and longitudinal neuropsychological studies [63, 64], show an accelerated rate of memory decline relative to noncarriers that predates symptomatic presentation of MCI or AD.

Lewy bodies, intracytoplasmic fibrillar aggregates of a-syn and ubiquitin, are a signature feature of Parkinson’s disease (PD), and a defining feature of Dementia with Lewy Bodies (DLB) [65]. The synucleinopathies are a group of disorders that include PD, DLB, and Multiple System Atrophy (MSA) [65]. Dementia commonly occurs within the context of parkinsonism, but is an infrequent accompaniment of MSA. Neuropathological studies have shown that cortical Lewy bodies are a common feature in patients with PD (32% with H&E stains, up to 76% with ubiquitin stains), and a defining feature in the setting of concurrent parkinsonism and dementia [66]. Distinctions currently made between “Parkinson’s Disease with Dementia” (PDD) and DLB are based on the duration of parkinsonism preceding the onset of cognitive symptoms, but neuropathological findings are similar whether dementia occurs as an early or late feature. Dementia itself correlates with cortical Lewy body burden whether or not there is concurrent AD pathology [67]. Distinguishing the relative contributions to dementia of lewy body and AD pathology in PDD and DLB patients is difficult. More than half of patients with clinically diagnosed DLB have AD pathology in addition to neocortical Lewy bodies at autopsy [68], and among patients with clinically diagnosed AD, neocortical Lewy bodies are found in 15% at autopsy [69]. Clearly additional tools to facilitate diagnoses of neurodegenerative diseases, particularly early in the course of the diseases would be very beneficial. Thus, there is a need to develop such tools that can be used for the diagnosis of neurodegenerative diseases and as adjuncts to therapeutic regimens.

**SUMMARY OF THE INVENTION**

In the present invention, the inventors have developed an electronic biosensor that can simultaneously determine not just levels of these key protein biomarkers, but levels of specific key morphologies of each of these critical proteins. Selected biomarkers have already been identified to help diagnose some of these diseases and these can be readily incorporated into the biosensor as well, however because these diseases are connected with aggregation of specific proteins, detecting levels of selected forms of each protein will enable an earlier and more accurate diagnosis of the specific neurological disease and provide a means to follow therapeutic strategies to determine their efficacy. The biosensor is designed so that it can be ultimately used in a physician’s office using serum or CSF samples.

More specifically, the invention describes a biosensor comprising:

- a microelectrode array base platform containing an array of conductive-material sensing sites, wherein each sensing site is comprised of a working electrode (WE) and a counter electrode (CE) wherein the surface area ration of CE:WE is between about 20:1 and 300:1;
- a nanoporous membrane overlaid on the platform wherein the membrane forms nanowells wherein each nanowell comprises immobilized therein an antibody agent that specifically detects a marker of a neurodegenerative disease;
- a microfluidic encapsulant to enable lateral flow of reagents over and into the nanowells.

In certain embodiments, the conductive material is platinum, gold, silver, or copper. In certain embodiments, the array comprises circular conductive-material sensing sites. In certain embodiments, the ratio of CE:WE is 50:1. In other embodiments, the ratio of CE:WE is 200:1. In certain embodiments, the nanoporous membrane is aluminum. In certain embodiments, the nanowell has an effective diameter of about 10 nm to 500 nm. In certain embodiments, the nanowells are rectangular, hexagonal, circular, elliptical, or other shape.

In certain embodiments, the biosensor further comprises a readout amplifier electrically connected to nanowires in one or more regions of the membrane. In certain embodiments, the biosensor further comprises a fluid chamber. In certain embodiments, the fluid chamber is formed of polydimethoxy silane (PDMS). In certain embodiments, the base platform is an insulator or includes an insulator portion. In certain embodiments, the base platform is silicon, glass, fused silica, polycarbonate, polyamides, ceramics, epoxy, or plastic, and may further comprise an oxide layer.

In certain embodiments, the biosensor further comprises one or more conductor strips coupling the biosensor to a sensor, wherein the sensor is coupled to a multiplexer, and the multiplexer may be controlled by a computer.
[0021] In certain embodiments, the marker of a neurodegenerative disease is an Aβ, a-syn or tau morphology. As used herein, the term “antibody” includes scFv (also called a “nanobody”), humanized, fully human or chimeric antibodies, single-chain antibodies, diabodies, and antigen-binding fragments of antibodies (e.g., Fab fragments). In certain embodiments, the antibody agent is an antibody, an Fab, or a nanobody. In certain embodiments, the antibody agent is a C6, A4, E1, D5, 10H, 6E, D10 or BSECI nanobody.

[0022] In the biosensor of the invention the marker of a neurodegenerative disease is a nanobody that detects to selected Aβ, a-syn and tau morphologies. For example, the biosensor has immobilized thereon a C6 nanobody. In certain embodiments, the C6 nanobody is less than 300 amino acids in length and comprises amino acid residues 16-292 of SEQ ID NO:1. In specific embodiments, the antibody fragment has an amino acid sequence of SEQ ID NO:1. The antibody fragment is specific for a 12-16 kDa oligomeric species of Aβ.

[0023] The present invention discloses an antibody or antibody fragment that specifically recognizes oligomeric Aβ that is at least partially resistant to denaturation by SDS (i.e., is SDS-stable) but does not bind monomeric or fibrillar Aβ or in vitro-generated oligomeric Aβ. Also contemplated herein is a binding molecule that binds to oligomeric Aβ that is at least partially resistant to denaturation by SDS but does not bind monomeric or fibrillar Aβ or oligomeric forms of Aβ that are generated in vitro, wherein the binding molecule comprises the sequence of SEQ ID NO:1. As used herein, “SDS-stable” means that the oligomeric aggregate does not dissociate into the monomer units in SDS (such as in 1% SDS).

[0024] The present invention also provides a biosensor device comprising a solid substrate and immobilized thereon one or more of a C6, A4, E1, D5, 10H, 6E, D10 or BSECI nanobody.

[0025] Also contemplated is a method of detecting the presence of a neurodegenerative disease comprising contacting a biological fluid from a subject suspected of having a neurodegenerative disease with a biosensor of the invention and measuring the output impedance of said biosensor in the presence of said biological fluid wherein a change in the measured output impedance across a sensing site is indicative of the presence of a marker of a neurodegenerative disease.

[0026] The present invention further provides a biosensor device comprising: (a) a printed circuit board platform, (b) a nanoporous membrane, and (c) a micro fluidic chamber. In certain embodiments, the printed circuit board platform comprises inter-digitated working and counter electrodes. In certain embodiments, the electrodes are tin oxide, gold, platinum, silver, or copper, or a combination of these materials, such as tin oxide and gold. In certain embodiments, the electrodes are about 600-1000 μm in width, about 2-10 mm in length and about 600-1000 mm in thickness. In certain embodiments, the electrodes are about 800 μm in width, about 5 mm in length and about 800 nm in thickness. In certain embodiments, the electrodes have rounded edges to minimize fringe effects during the application of a sinusoidal voltage input signal. In certain embodiments, the nanoporous membrane is soldered onto the interdigitated electrodes generating a high density array of nanowells. In certain embodiments, the nanoporous membrane is attached to the interdigitated electrodes by means of epoxy bonding, pressure attachment. In certain embodiments, the nanoporous membrane is flash on the substrate. In certain embodiments, the nanoporous membrane is a nanoporous alumina membrane.

In certain embodiments, the nanoporous membrane is poly-carbonate, a metal oxide, or a ceramic-based material. In certain embodiments, the nanoporous membrane is 100-500 nm thick, has a lateral diameter of 5-20 mm with pore diameters of 100-500 nm. In certain embodiments, the nanoporous membrane is 250 nm thick, has a lateral diameter of 13 mm with pore diameters of 200 nm. In certain embodiments, the nanoporous membrane has a porosity of about 25% to 50%. In certain embodiments, the micro fluidic chamber is silicone. In certain embodiments, the micro fluidic chamber is acrylate, plastic, or any other biocompatible hydrophilic material. In certain embodiments, the micro fluidic chamber forms an enclosure for the printed circuit board platform and the nanoporous membrane to prevent evaporation. In certain embodiments, the micro fluidic chamber has a volume of about 10 μL to about 5 mL. The volume needs to be sufficient to wet the membrane and not dry out. In certain embodiments, the micro fluidic chamber has a volume of about 1.6 mL. In certain embodiments, the electrode is contacted with a negatively-charged substance to form a coated electrode surface. In certain embodiments, the charged substance is an amine (e.g., 3-aminopropyl triethoxysilane (APTES)) or BST. In certain embodiments, the biosensor device further comprises an antibody agent immobilized onto the printed circuit board platform or the coated electrode surface. In certain embodiments, the antibody agent is an antibody, an Fab, or a nanobody. In certain embodiments, the antibody agent is immobilized onto the printed circuit board platform or the coated electrode surface by means of one or more negatively- or positively-charged chemical linkers. In certain embodiments, the chemical linker is a thiol linker, a carboxylic linker, or a hydroxy linker. In certain embodiments, the thiol linker is 3,3′-dithiobis succinimidyl propionate (DSP).

[0027] The present invention further provides a method of making a biosensor device comprising:

(a) functionalizing an electrode surface with an amide to form a silanized electrode surface,
(b) solddering an alumina membrane to the silanized electrode surface,
(c) coating the silanized electrode surface with a cross-linking agent to form a functionalized surface, and
(d) contacting an antibody agent to the functionalized surface.

BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS

[0032] FIGS. 1A-1F. Nanodevice of the invention. (A) Nanosensor (NM) device, (B) Sensing Site—Working (WE) and Counter (CE) electrodes, (C) Input/Output pads connecting to sensing site through interconnects, (D) Nanoporous membrane overlaying on NM, (E) SEM of membrane, and (F) the microfluidic encapsulant fabricated out of biocompatible acrylic to regulate the lateral flow of reagents onto the sensing sites. The encapsulant is interfaced to the device through a pressure sensitive adhesive. It consists of 8 microchannels, each encapsulating one sensing site and consisting of an input and output port. Each microchannel supports the flow of 8 μl onto the sensing site.

[0033] FIG. 2. Binding specificity of nanobodies toward different oligomeric Aβ forms by time course immunoactivity assay. Aβ40 was incubated for 10 days and aliquots removed and probed with E1 and A4 scFvs.
FIG. 3. E1 binds human AD but not healthy brain extract. Brain extract from non-disease (ND) and AD patients were probed with E1 nanobody.

FIG. 4. AFM images of 3 different anti-oligomeric Aβ phage displayed sScFs. Phage appear as long thin filaments. C6 reacts only with brain derived oligomers, E1 with 1 day synthetic oligomers, and A4 with 3 day synthetic oligomers.

FIG. 5. Detection of oligomeric a-syn in human post-mortem CSF samples on Nanomonitor devices. The D5 nanobody against oligomeric a-syn was immobilized into the nanowell surfaces of the sensing sites. CSF samples were injected onto the sensing sites. Binding of the oligomeric a-syn was characterized by measuring impedance changes from the baseline. A minimum of three replicates were performed for each sample, and the data was extracted for the 100 Hz frequency.

FIGS. 6A-6B. Detection of oligomeric a-syn (A) and Aβ (B) in human post-mortem CSF samples using electronic biosensor. The nanobodies D5 (oligomeric a-syn) and A4 (oligomeric Aβ) were separately immobilized onto nanowell surfaces. CSF samples were injected and binding of specific oligomeric a-syn or Aβ species was determined by measuring impedance changes from baseline. At least three replicates were performed for each sample. Data was extracted for 100 Hz frequency. Three CSF samples of Non-demented (ND1-3), AD (AD 1-3), and PD (PD1-3) cases were analyzed. PD3 represents an MSA patient with Parkinsonism.

FIG. 7. Illustrates conductive strips 403-406 with gaps 402 formed on a surface of a base substrate 400.

FIG. 8. Provides nucleic acid and amino acid sequences for several nanobodies. Underlining indicates CDR regions (or nucleic acids that encode CDR regions).

FIGS. 9A-9D. (A) Physical layout of the sensor platform on PCB surface (B) Integration of the alumina membrane (C) Incorporation of silicon chamber and (D) Changes to the electrical double layer within each nanowell due to the binding of the target antigens onto the nanobodies.

FIG. 10. Impedance spectrum variation over the frequency range from 50 Hz to 1 MHz. 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. 11. 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.

FIGS. 12A-12C. 4 (A) Normalized data with D5 antibody (B) Normalized data with A4 antibody (C) Qualitative representation of the data.

DETAILED DESCRIPTION OF THE INVENTION

Biosensors are very powerful tools for the detection of biological markers or targets in samples obtained from media such as blood and tissue. The successful coupling of electrochemistry with naturally existing sophisticated biomolecules has enabled the design of novel, real time molecular recognition technologies that are both extremely sensitive and selective. The inventors have developed a NanoMonitor biosensor that can detect low concentrations (femtomolar) of multiple target antigens in biological fluids such as serum and CSF [70-72]. The high sensitivity the Nanomonitor combined with the high specificity of morphology specific nanobodies provides a unique opportunity to determine how serum and CSF levels of individual aggregate species of Aβ, a-syn and tau correlate with various different neurodegenerative diseases.

A biosensor that can accurately distinguish between different neurodegenerative diseases and facilitate pre-symptomatic diagnoses would be an extremely valuable clinical tool. It is shown here that it is possible to construct a simple, robust, biosensor that can be utilized in a physician’s office to sensitively differentiate between AD, PD, DBL and other neurodegenerative diseases. Substantial efforts have been expended to identify biomarkers that can distinguish between these diseases (see for example: [73-75]). The most promising CSF biomarkers identified are Aβ, tau and phosphorylated tau levels, however in general studies employing these biomarkers have met with somewhat limited success [76]. There are several unique aspects that distinguish this approach from similar previous studies and strongly increase our chances of succeeding. One major difference is that the reagents to be utilized in the biosensor can selectively identify specific toxic forms of individual proteins that are correlated with disease progression. The presence of specific oligomeric Aβ aggregate species has been correlated with AD, the presence of tau aggregates has been correlated with AD and other tauopathies, and the presence of a-syn aggregates has been correlated with PD, DBL and other synucleinopathies.

The present invention uses a set of reagents that can selectively identify different Aβ aggregate species, and another set that selectively recognize different a-syn species. The invention also can be used with reagents that will recognize different tau species. The inventors have generated and characterized recombinant antibody fragments or nanobodies against specific protein morphologies that can be used in the biosensors. In order to isolate such nanobodies to individual aggregate forms, a novel AFM biopanning technology was used that enabled the inventors to visualize the target protein morphology and monitor the panning process [52]. Utilizing this technology nanobodies were isolated that recognize different areas of monomeric Aβ and a-syn [77-80], fibrillar Aβ and a-syn [52, 81], two different oligomeric a-syn species [48, 51], two a-syn/dopamine adduct species, and three different oligomeric Aβ species [82]. The different oligomer specific nanobodies do not show cross-reactivity, so the nanobodies binding oligomeric Aβ do not bind oligomeric a-syn and vice versa. Each of the different aggregate species recognized by the different nanobodies naturally occur in human AD or PD tissue, and that the nanobodies can be used to distinguish between AD, PD and healthy brain tissue, and block toxicity of different aggregate species [48, 51, 82]. Therefore the inventors have a unique panel of well characterized and highly selective reagents that can be used to correlate the levels of key aggregated protein forms associated with different neurodegenerative diseases.

This panel of reagents is used in combination a label-free biosensor technology that operates on the principle of electrochemical impedance spectroscopy that is ideally suited to identify protein biomarkers in clinical samples. Thus, the panel of nanobodies is used in combination with the NanoMonitor assay (US patent application number: 20070256941 incorporated by reference herein) as a clinical diagnostic tool for simultaneous detection of low nano- and picogram/ml levels of various target antigens in patient serum samples [70-72]. The morphology specific reagents in con-
junction with the Nanomonitor can distinguish between PD, AD and healthy human CSF samples. The biosensor has several features that make it 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 Nanomonitor assay can determine antigen concentrations over large concentrations ranges with detection limits down to low femtomolar or even attomolar levels.

Electrochemical devices are extremely useful for delivering the diagnostic information in a fast, simple, and low cost fashion, and are thus uniquely qualified for meeting the demands of point-of-care screening for a variety of diseases. In particular, Electronic Impedance

Spectroscopy (EIS) offers an elegant way for interfacing biomolecular recognition events with electrochemical signal transduction for simple, rapid, and label-free detection of ultralow concentrations of biomarkers for disease. The remarkable sensitivity of such electrochemical sensing protocols opens up the possibility of detecting disease markers that cannot be measured by conventional methods and has potential applications for early detection of disease [83].

The Prasad lab has designed and developed a label-free biosensor technology operating on the principle of electrochemical impedance spectroscopy suitable for protein biomarker profiling from clinical samples. This technology known as the NanoMonitor assay (US patent application number: 20070256941) has been demonstrated as a clinical diagnostics tool in detecting C-reactive protein and Myeloperoxidase (protein biomarkers associated with vulnerable coronary vascular plaque) from patient serum samples collected through a pilot study conducted in collaboration with Oregon Health Sciences University and the Portland Va. at clinically relevant concentrations in the lower picogram/ml range [70, 71]. The NanoMonitor assay was also tested for detecting allergens such as Japanese tree pollen (rye j) from serum samples at clinically relevant concentrations in the nanogram/ml regime [72]. The notion of antibody confinement in nanoscale arrays for enhancing the antibody density and enhancing antibody-antigen interactions has been the genesis for designing the nano-template based NanoMonitor. In comparison to other electrochemical diagnostics assays currently under development, the NanoMonitors have a significant advantage as they provide demonstrate simultaneous detection of multiple protein biomarkers at clinically relevant concentrations from complex samples such as purified human serum with comparable sensitivity and specificity as that observed for synthetic samples in isotonic buffers [70-72].

The NanoMonitor is comprised of three parts: the microelectrode array base platform, a nanoporous alumina membrane overlay which forms the nanowells, and the microfluidic encapsulant to enable the lateral flow of reagents over and into the nanowells. The microelectrode base platform contains an array of circular gold measurement/sensing sites where the binding of the protein molecules occurs in a controlled manner (FIG. 1A). Each sensing site is comprised of a working electrode (WE) — and a counter electrode (CE) (FIG. 1B), wherein the surface area ratio of the CE to WE is 225:1 (FIG. 1B). Additionally the distance of separation between the circular portions of the WE and CE is very small (2 μm). The capacitance change due to biomolecule binding is measured across the WE and CE. Based on fundamental principles of electrical engineering, the capacitance change is maximized by an increase in the differential surface area and a decrease in the distance of separation between WE and CE. The metal electrodes function as the base for multiple nanowells once the nanoporous alumina membrane is overlaid. Protein binding and capacitance changes occur at these electrodes (sensing sites). These electrodes are connected, to input/output measurement leads. An input low voltage alternating current (AC) signal is provided to the sensing site through these leads and the output capacitance signal is measured across the same leads (FIG. 1C). Both the electrodes are designed to be circular in shape to attain maximum surface area of interaction and to avoid any possible edge effects causing impedance variations. The microelectrode is designed to get 8 such sensing sites on a 20x20 mm² silicon chip using the standard process of photolithography used for fabricating microelectronics. The second part of the nanomonitor is the nanoporous alumina membrane (FIGS. 1D and 1E). A commercially available nanoporous alumina membrane with nanopore diameter of 200 nm and membrane thickness of 250 nm (Anodisc, Watman, N.J., USA) is overlaid and adhered using a non-toxic epoxy glue onto the base microelectrode array [70]. The pore diameter is selected such that the pore is large enough to function as a scaffold for one or more antibody-antigen binding complexes. At 200 nm diameter there are approximately one quarter million nanowells on a single sensing site [119]. The third part is the microfluidic encapsulant fabricated out of biocompatible acrylic (FIG. 1F) to regulate the lateral flow of reagents onto the sensing sites. The encapsulant is interfaced to the device through a pressure sensitive adhesive. It consists of 8 microchannels, each encapsulating one sensing site and consisting of an input and output port. Each microchannel supports the flow of 8 μl onto the sensing site.

Thus, in certain embodiments the invention uses nanoporous membranes in which the aggregate detecting nanobodies are mobilized. The skilled person will be aware of various materials that can be used to form nanoporous membranes. The nanopores in the membrane can all be of a uniform size, arranged in a series of rows and columns, but other arrangements of pores of the same or different sizes can be used. Such nano-pores can be arranged in regular patterns, irregularly, or can be randomly distributed. The nano-pores can be of similar sizes (length, cross-sectional area), or different sizes or a selected distribution of sizes can be provided in a single substrate. Nano-pores typically have cross-sectional areas similar to the cross-sectional areas of cylinders having diameters of between about 5 nm and 1000 nm. Nano-pore aspect ratios (length/diameter) typically are in a range from about 0.5 to 1000 Dimensions, shapes, and aspect ratios can be selected based on a particular application. Nano-pore cross-sectional areas can be rectangular, hexagonal, circular, elliptical, or other shape. Nano-porous membranes can be used for specimen analysis based on, for example, sample size and structure (size based filtration) or based on nano-pore sensitization using antibodies or other sensitizing agents.

Nano-pores in one or more regions of the membrane are electrically connected to a readout amplifier, typically a differential amplifier that can produce a signal based on a difference in an electrical characteristic of the nano-pores in the different regions. The electrical readout can be processed to obtain, for example, a spectrum (using, for example, a fast
Fourier transform), a power spectral density, or to identify a particular spectral component associated with an intended response. The electrical readout can be configured to permit measurement of a time evolution of response so that, for example, spectrum as a function of exposure time is determined.

[0054] Substrates are generally selected for ease of nanopore formation. Aluminum is convenient as it can be electrochemically processed to produce alumina nano-pores of hexagonal cross-sectional area, and having different aspect ratios. Aspect ratios (length/diameter) of at least about 1000 : 1 can be achieved. Aperture dimensions can be configured based on electrochemical bath temperature and composition, applied voltage, current density, and/or exposure duration. Different aperture dimensions can be provided on a single substrate by selectively processing different substrate regions. Different size pores can be particularly useful in sized-based protein trapping in which the response of different pore sizes can be associated with protein size or other analyte property. For electrical measurements, the substrate is preferably substantially non-conductive, although configurations in which the nano-pores are electrically isolated from the substrate can be used as well. Substrates such as silicon, silicon oxides and nitrides can also be used, and apertures can be formed by wet or dry etching, ion beam milling, or other process. Surface portions of the substrate can be coated with a conductive material such as platinum, gold, silver, copper, or other material by sputtering, evaporation, or other processes so as to electrically couple a plurality of nanopores forming sets of nanopores. In typical examples, antibodies or other sensitizing agents are immobilized on surfaces of the nanopores, typically nanopore sidewalls.

[0055] In representative examples, sensors comprise a substrate having defined therein at least one nanoporous membrane portion that includes a plurality of nanopores. The nanopores are electrically coupled with a sensing site. In preferred embodiments, the sensing site is comprised of a working electrode and a counter electrode wherein the surface area of the CE: WE is between about 20:1 to about 500:1. It has been found by the inventors that 20:1 and 50:1 ratios have a much better sensitivity than 10:1 ratio. In specific embodiments, there is a plurality of nanopores and each nanopore is electrically coupled to sensing site. Alternatively, a plurality of nanopores can be electrically coupled to one sensing site. In addition, it is contemplated that there are different sensitizing agents (by sensitizing agents, the present invention means morphology specific nanobodies that are use to determine the presence of aggregate species of Aβ, a-syn and tau) coupled to different nanopore sites such that there is a first sensor either coupled to one nanopore or a plurality of nanopores each having immobilized thereon the same sensitizing agent and a further second, third, fourth etc, sensor that is either coupled to one nanopore or a plurality of nanopores each having immobilized thereon the same second, third, fourth etc, sensitizing agent.

[0056] In preferred embodiments, the sensing site in the present invention comprises of circular gold sensing sites. However, it should be understood that the gold is simply a conductive material for the sensing site and may be replaced with other materials such as platinum, silver, copper, and the like.

[0057] In specific embodiments, a spectrum analyzer is in communication with the sensing sites and is configured to produce an estimate of a received signal portion associated with a signature frequency or frequencies. In additional examples, the spectrum analyzer is configured to produce an estimate of a received signal portion associated with at least two frequencies associated with a first signature and a second signature.

[0058] In operation, a test specimen of a fluid from a subject to be diagnosed is contacted with a biosensor of the invention, and evaluating an electrical signal associated with administration of the test specimen to the nanoparticles. The test specimen can be assessed based on the evaluation. In some examples, the electrical signal is evaluated to identify a magnitude at least one electrical spectral peak associated with exposure of the sensitized nanopores to the target compound, and the test specimen is assessed based on the magnitude. In further examples, the electrical signal is evaluated to identify an electrical signature associated with the target compound, and the test specimen is assessed based on the signature.

[0059] Thus, as noted above, the biosensor of the invention has three components: a base substrate containing an array of electrically conductive materials that are linked to nanoparticles in a nanoporous membrane and a third component which is a fluid chamber that allows channels to form to produce a lateral flow of reagents over and into the nanopores. The fluid chamber includes an inlet port and an exit port and is situated so that the nanowells in the nano-membrane are exposed to reagents provided to the fluid chamber through the inlet port. Fluid chamber volume can be selected based on, for example, a convenient specimen volume, and is typically between about 1 μl and 1000 μl. Conductor strips are provided on the base substrate, and are electrically coupled to respective portions of a second surface of the nano-membrane. The nanomembrane includes a plurality of nanopores that couple the first and second surfaces.

[0060] In a convenient example, the nano-membrane is an alumina membrane formed from an aluminum foil, and gold conductor strips are patterned and formed on the base substrate using contact photolithography. Other membrane materials can be used, and conductors of silver, gold, copper, or other conductor OP semi-conductor materials can be used. The fluid chamber is formed of polydimethoxysilane (PDMS), but other materials can be used. Alternatively, the chamber can be omitted and test materials dispersed directly onto the first surface of the nano-membrane.

[0061] The nanoporous membrane will have therein a plurality of nanowells having effective diameters of about 10 nm to 500 nm. The pores can have circular, elliptical, hexagonal, cross-sections, or cross-sections of other shapes. In certain applications, pore diameter is substantially uniform or variable within a predetermined range. The nano-membrane is preferably an electrical insulator so that the pores are not electrically coupled to each other except addition electrical connections such as the conductor strips.

[0062] The base substrate is generally an insulator, or includes an insulator portion. For example, silicon with an oxide layer can serve as the base substrate, wherein the conductor strips are defined on or in the oxide layer so as to be substantially electrically isolated. Such a base substrate can be especially convenient for inclusion of detection electronics in the base substrate. However, other substrate materials such as glass, fused silica, polycarbonate, polyanides, ceramics, epoxy, plastics, or the like can be used.

[0063] For example, the base substrate is formed using a 2 cm by 2 cm section of silicon wafer cleaved from a larger wafer. This substrate is cleaned in piranha solution, spin
coated with a positive photoresist, and a quartz photomask is used to define features 1 μm by 2 cm. A 10 nm thick gold film is sputtered coated onto the photoresist, and gold conductor strips 2 μm by 2 cm can be formed using a lift-off process. FIG. 7 illustrates conductive strips 403-406 with gaps 402 formed on a surface of a base substrate 400.

[0064] Alumina membrane fabrication is known to those of skill in the art. High purity aluminum foil substrates (99.99% pure) are selected and sized, degreased in acetone, and cleaned in an aqueous solution of HF, HNO₃, and HCl in a volume ratio of about 1:1:2.5. After cleaning, the substrates are annealed in a nitrogen ambient at 400 °C for about 45-60 min. to remove mechanical stresses and allow re-crystallization. Grain sizes can be measured using electron microscopy, and grain sizes in the annealed substrates are typically between about 100 nm and 200 nm. Surfaces of the annealed substrates are electro-polished in a mixture of HClO₄ (perchloric acid) and C₂H₅OH (ethanol). The substrates can be anodized at a constant cell potential in aqueous H₂SO₄ (sulfuric acid) at concentrations of between about 1.8 M and 7.2 M. Sulfuric acid/oxalic acid mixtures can also be used. Typical mixtures are combinations of 0.3 M oxalic acid with 0.18 M to 0.5 M sulfuric acid. Current densities typically range from about 50-100 mA/cm².

[0065] Multi-step anodizations can also be used. In a typical two-step anodization, a first step is used to form a concave texture and a second step is used to form nanostructures, typically at locations at which texture changes were formed in the first step. In a typical first anodization, the aluminum substrates are mounted on a copper plate anode, and a graphite plate is used a cathode. During anodization, the electrolyte is vigorously stirred and/or recycled, and cell voltage, current, and temperature are monitored and recorded. In this first anodization, cell potential is fixed at about 40 V and the substrate is exposed to 0.3 M oxalic acid (H₂C₂O₄) electrolyte solution for about 3 hrs at about 25°C. In a second anodization, partially anodized substrates are exposed to a mixture of 6% by weight of phosphoric acid and 1.8% by weight of chromic acid for about 10 hrs at a temperature of about 60°C. After this second anodization, the first anodization is repeated for about 5 hrs. Pores are generally about 20 nm wide and about 25 nm deep. Any remaining aluminum in the substrates can be removed with a saturated mercuric chloride solution.

[0066] For anodization, an aluminum substrate is secured to a copper plate that serves as an anode. A graphite plate is used as a cathode, and the aluminum substrate/copper plate and graphite plate are exposed to an electrolyte solution at a selected applied voltage. Electrolyte solution temperature, composition, and concentration, and applied voltage are selected to provide an intended pore size, aspect ratio, and/or pore density.

[0067] In typical examples, nanopores having diameters of about 25, 50, and 100 nm are produced using cell voltages of about 12 V, 25 V, and 40 V, respectively, at a cell temperature of about 60°C. Current density varies from about 1.2 A/cm² to 5 A/cm². Pore densities can be varied from about 610⁹/cm² to about 510¹⁰/cm², and are typically directly proportional to current density and inversely proportional to cell temperature.

[0068] In the second anodization step, varying the electrolyte temperature from 25°C to 50°C. In increments of 1°C, for every 10 minutes permits selection of pore widths in a range of about 12 nm to 200 nm. Varying the applied voltage from 40 V to 70 V at 5 V increments every 10 minutes permits selection of pore surface density in a range of about 10⁶ pores/mm² to 10⁸ pores/mm², and pore depth can be altered from about 10 nm to 250 nm by increasing the voltage. By varying the concentrations of oxalic, phosphoric and chromic acids from about (1.0:0.5:0.5) by volume to about (2.5:3:3) by volume, pore width can be varied from about 12 nm to 750 nm.

[0069] Pores typically nucleate on the surfaces of the substrates at approximately random locations, and pores have random locations and a broad distribution of sizes. Under certain specific conditions, a hexagonal ordering of pores is produced. These pores are well suited for trapping of nanometer sized particles. Pore sizes for a particular application can be selected based on a protein size so that the target protein “fits” the pores. Such a cut can reduce non-specific binding events, increasing measurement sensitivity and reliability.

[0070] Sensors can be interrogated by coupling one or more conductor strips. A sensor includes a plurality of conductors that are coiled to a multiplexer that selects one or more of the conductors for coupling to a buffer amplifier. The multiplexer can be controlled for such selection based on a user selection or under control of a desktop, laptop, or palm top computer. Alternatively, each conductor can be coupled to a respective buffer amplifier, and signals on all conductors made simultaneously available for signal analysis. In other examples, a mechanical switch or probe can be used to selectively couple to one or more conductors.

[0071] The conductors can be associated with different sensitivities (for example, contacted to nano-pores on which different types of antibodies are immobilized). Electrical signals from the conductors are based on, for example, effective conductance variations associated with binding of antigen-antibody complexes. These electrical signals exhibit complex time domain behavior, but generally have characteristic features or “signatures” when viewed in the frequency domain. Typically, a specific bound complex is associated with one or more characteristic frequencies, and signal magnitude at the characteristic frequency (or frequencies) is a function of analyte concentration.

[0072] Characteristic frequencies can be detected with a spectrum analyzer that is coupled to the selected conductor (or conductors) and that receives an electrical signal associated with the sensitized conductors/nano-pores. The spectrum analyzer can be implemented using a mixer and a swept oscillator with a detector that is coupled to evaluate a magnitude and/or phase of a difference or sum frequency from the mixer. Alternatively, a time record of the coupled electrical signal can be stored, and a spectrum obtained using, for example, a fast Fourier transform. In some examples, a power spectrum is obtained in order to identify presence of a targeted material, or a response to a compound under investigation. A differential electrical signal is generally used such that a difference signal associated with a reference conductor and a conductor coupled to sensitized nano-pores is evaluated. Signals are generally available within seconds after exposure of a sensitized membrane to an analyte, and thus permit rapid analyte assessment. A signature analysis processor is generally coupled to receive the detected spectra and, based on signatures stored in a signature database, determine presence and/or concentration of one or more analytes.

[0073] Key to the biosensors of the present invention is that they employ antibodies that detect neuropathies such as α-syn and tau morphologies. Binding of the antigen aggre-
gates to these antibodies results in a detectable variation in signal. Detected voltage variations are based on binding of the antibody-antigen protein complex to a base substrate.

[0074] Aβ, tau and a-syn are proving to be critical components in the etiology of several devastating neurodegenerative diseases including AD, PD and D LB. Extensive studies show that accumulation and aggregation of these different proteins are connected with various AD, PD, DLB, and other synucleinopathies and tauopathies. Accumulation of soluble Aβ, for example, has been correlated with the severity of AD [8] and is thought to lead to diffuse plaque formation, setting off a cascade of events including activation of microglial cells, inflammation and misprocessing of tau, which results in the other dominant pathological feature of AD, neurofibrillary tangles (reviewed in [86]). Soluble oligomers of Aβ were shown to be cytotoxic, disrupt neuronal functions including LTP and learning, and correlate better with progression of AD and have been identified in AD brains (reviewed in [87]). Since cellular stress can lead to an increase in protein misfolding, it is likely that toxic aggregated species of Aβ can not only lead to misprocessing and aggregation of tau, but also to aggregation of a-syn and other proteins. Similarly aggregation of a-syn in PD can lead to misprocessing and aggregation of Aβ and tau. Therefore, well defined highly specific reagents that can identify and quantify individual morphologies of Aβ, a-syn and tau will be a valuable tool for use in biosensors to assist in early diagnosis of various neurodegenerative diseases.

[0075] Thus the sensitizing agents used in the biosensing devices of the invention are nanobody reagents specific for a variety of different morphologies of key proteins involved in neurodegenerative diseases including Aβ and a-syn. A single chain antibody variable domain fragments (scFvs or nanobodies) was isolated that specifically recognize monomeric [77], fibrillar [52], and two different oligomeric a-syn morphologies, one against SDS stable trimers and hexamers [51], and one binding dimers and tetramers [48]. The anti-oligomeric a-syn nanobodies do not cross react with oligomeric Aβ, and specifically label PD brain tissue but not AD or healthy tissue [51]. Dopamine interacts with a-syn to form stable toxic adducts, and nanobodies that specifically recognize monomeric and oligomeric a-syn/dopamine adducts, but not monomeric or oligomeric a-syn alone (Table 1) were isolated. Therefore, the devices of the invention may use nanobodies that specifically recognize six conformationally distinct a-syn aggregate morphologies.

![Table 1](image)

<table>
<thead>
<tr>
<th>Clone</th>
<th>Control Oligomeric a-syn Dopamine alone Oligomeric a-syn/dop</th>
<th>Oligomeric a-syn/dop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olad A7</td>
<td>0.075 0.153 0.097 0.066</td>
<td></td>
</tr>
<tr>
<td>Olad C12</td>
<td>0.056 0.050 0.040 0.018</td>
<td></td>
</tr>
</tbody>
</table>

[0076] In addition, nanobodies to different regions of monomeric [79, 80] and fibrillar Aβ [81] also may be used. The inventors also isolated a nanobody (A4) that specifically recognizes oligomeric Aβ, does not cross react with oligomeric a-syn, and specifically labels Aβ aggregates in human AD brain samples, but not PD or healthy brain tissue [82].

[0077] In addition, the biosensor may have attached a nanobody (C6) that specifically recognizes Aβ dimeric species derived from human AD brain tissue [13, 87]. The different specificities of the three different anti-oligomeric Aβ nanobodies, A4, E1 and C6, can be readily visualized by AFM when the nanobody fragments are expressed on the surface of phase instead of as soluble proteins. Using this technique, the C6 nanobody can be seen to bind brain derived oligomeric Aβ, but not in vitro generated Aβ oligomeric species, while E1 binds the 1 day preaggregated in vitro sample containing tetrameric Aβ, but not the brain derived Aβ particles or the 3 day preaggregated in vitro Aβ sample, and A4 binds the 3 day preaggregated in vitro Aβ species, but not the 1 day or brain derived samples (FIG. 4). Each of these oligomeric forms occurs in human AD brain tissue as C6 binds to Aβ oligomers recovered from brain tissue and A4 [82] and E1 (FIG. 3) bind AD but not healthy brain tissue. There are three different nanobodies (A4, C6, and E1) that recognize three conformationally distinct small oligomeric Aβ species, and do not cross react with oligomeric a-synuclein aggregates. Thus there is a pool of nanobodies to six different aggregate morphologies of a-syn and five different morphologies of Aβ that distinguish between diseased and healthy brain tissue.

[0078] While Aβ, a-syn and tau are normally present in CSF at low nanomolar concentrations [21, 22], the in vivo concentrations of the various oligomeric forms of the proteins are likely considerably lower. Based on preliminary results showing that it is possible to obtain femtomolar sensitivity, it is contemplated that the monomeric binding nanobodies will have high enough affinity to detect the necessary concentrations in the serum and CSF samples. However, if higher affinity is required to detect a signal without background interference, the affinity can be readily improved by affinity maturation protocols. In order to increase the stability of the scFvs where necessary, the skilled person can convert the scFvs into the corresponding Fab2 form. Conversion is readily accomplished by splicing out the respective heavy and light chain variable domain regions and splicing them into vectors containing a constant heavy and light chain region, respectively. The resulting Fab2 fragments can then be produced using a bicistronic construct in E. coli. The protocols are standard and will be performed essentially as described [100].

[0079] The skilled person can also increase the affinity of a given nanobody for its target antigen or increase the specificity for target A over target B by affinity maturation. To do this, it is possible to sequentially randomize CDR regions from the antibody heavy and light chain regions to generate a secondary library of antibodies where the entire library is now based on antibody sequences specific for the target protein morphology. The first generation of this library will be constructed by randomizing the CDR3 region of the light chain essentially as described and subsequent generations will target other CDR regions as described [101]. Affinity maturation using antibody libraries has routinely improved the affinity of the parent antibody [101]-[106]. For increased specificity panning can be done for nanobodies that are specific only for target A oligomers by first performing a negative panning step to remove nanobodies that bind target B and C oligomers, and then recover nanobodies that are specific for target A oligomers. The inventors have successfully utilized this two step panning protocol to isolate nanobodies to a specific protein among a family of related proteins [107]. Such protein and morphology specific nanobodies will be extremely valuable for diagnostic applications to distinguish between different neurodegenerative diseases.
In certain embodiments, the biosensor is a detection assay such as an ELISA, Western blot, dot blot or the like.

Example 1

For the initial EIS experiments, nanobody fragments are covalently attached to thiols through Dithiobis [succinimidy] propionate) cross linker into the nanowells. Serial dilutions of purified nanobody are made in an isotonic buffer (15 mM phosphate buffered saline) and EIS spectra taken. The data is processed noting optimal frequency and/or equivalent circuit parameters for the given target to determine reproducibility and linear range of detection as well as limit of detection (LOD). The Gamry Ref 600 potentiostat is used to acquire the EIS spectra. This potentiostat system has built in Randles and Warburg models, which are used to determine the physical equivalent circuit element which is a measure of the nanobody binding to the sensor surface; double layer capacitance ($C_{dl}$) which is a measure of the binding of the nanobody fragments on to the sensor surface. Frequency analysis is also performed to verify optimal frequency for time based studies. Control samples, nonspecific antibodies or other targets are used to verify specificity and operation of the array.

Purified antigen samples are then be spik in 100% commercially available human serum to replicate the complex media of patient samples. Again, a linear range of concentrations of target is run as before to determine LOD, linear range, and reproducibility. Then the concentration of commercial human serum is diluted with buffer from 25%-70% and the study repeated. Comparisons of impedance at optimal frequency will be analyzed and plotted. The highest concentration of commercial human serum that still yields statistically significant results determines how much (if any) purification of the patient samples is required. The preliminary data shows that the antigens can be detected accurately with a linear dose range from 1 pg/ml to 1 pg/ml for 30% volume of human serum, hence the lowest dilution used is 30%.

The protocol for detecting the proteins is as follows: The nanowells on the sensing site are immobilized with nanobody through the DSP linker chemistry. The nanobody saturated nanowell surfaces are treated with a blocking agent to ensure that unbound linker surfaces are blocked. Proteins are then added onto the nanobody saturated substrate. Binding of proteins is monitored by changes to the impedance through electrochemical impedance spectroscopy. Protein dose response is performed by measuring impedance changes from the NanoMonitor due to changing doses of the protein. All the biomolecules (linker molecules, antibodies and proteins) have surface charges. The binding of these molecules to the base of each nano-well perturbs the charge distribution in the electrical double layer that forms at the solid/liquid interface. This charge perturbation produces a capacitance change in the electrical double layer. The biomolecule binding induced capacitance change is measured by the electrochemical impedance spectroscopy technique. In this technique protein binding to the sensing site is achieved through the immobilization linker specific antibodies to the nano-wells. A low voltage (50 mV-1V) AC excitation signal at very low frequency range (10 Hz to 1 kHz) is applied to a sensing site. Binding of the biomolecules produces a change in the measured output impedance across a sensing site. The measured impedance is the sum total of two components: the resistance and capacitance of the sensing site. It is only the capacitive component that is a measure of the biomolecule binding event, as the capacitive component indicates the surface charge differential in the electrical double layer as a function of biomolecule binding. Hence the input voltage parameters (frequency and voltage amplitude) need to be optimized to measure this capacitive component. The optimization range is focused to voltages below 0V as higher voltages have demonstrated hydrolysis of biomolecules and frequencies >1 kHz have been associated with impedances changes in solution bulk and not to the electrical double layer (EDL).

The inventors have demonstrated that they can obtain femtomolar sensitivity for the target antigen using the morphology specific nanobodies in conjunction with the NanoMonitor assay even in the presence of complex media. In addition, the inventors have also shown that they can detect the presence of specific target protein morphologies in complex media such as CSF.

The optimal signal frequency and amplitude (impedance), may vary for each nanobody and target, however it is expected that the linear signal range and limit of detection will be similar for most of the systems to be studied. It may be necessary, with the use of serum samples, to reduce background noise. One method to do this is to use mixed alkanethiol monolayers [34] that allow for binding of the capture element but block non-target molecules by use of nonfunctional thiols groups or other cappers. Additionally due to the viscosity of whole serum or whole blood clinical samples it is possible that there will be fouling of the nanoporous alumina preventing the transport of the antigens interest into the nanowells. By reducing the concentration of the whole serum, the limit of detection can be ascertained in the sense of how pure a sample is needed in order to maintain detectability.

Other noise reduction strategies including blocking and washing steps may also be required to reduce nonspecific binding effects particularly with serum samples. Different blocking agents, such as, bovine serum albumin (BSA), casein (an isolate from bovine milk), polyethylene glycol (PEG), ethanolamine, commercial combinations such as SuperBlock Blocking Buffer, etc. will be used at various concentrations (low, e.g. 0.5% or 1 mM to high levels 10% or 100 mM depending upon blocking agent) and for various times (ranging 1-120 min) An important component to the blocking step is the washing step. Temperature of the wash, component(s), and time and volume of rinses will all be investigated. Here, the expected outcome is that a limit of % of human serum will be reached where signal becomes either non-linear or altogether lost.

Protein Aggregation.

Soluble oligomers of Aβ, a-syn and tau are formed as transient intermediates during the aggregation of each protein. The inventors have extensive experience in studying aggregation of proteins involved in neurodegenerative diseases [48, 51, 77-82, 89-92] and can readily generate a variety of different Aβ aggregates. Since the inventors already have nanobodies to several oligomeric Aβ forms generated either at neutral pH or isolated from brain tissue, here the inventors will focus on generating Aβ aggregates at acidic pH. Since Aβ may be generated at least in part by proteolytic processing in endosomes, some Aβ aggregates may originate under these acidic conditions. To do this, the inventors will aggregate Aβ essentially as before, but use a sodium acetate buffer (pH 4.5) instead of PBS buffer (pH 7.4). The C-terminal truncated species of a-syn are abundant in Lewy body deposits, aggregate more rapidly than the full length form [93, 94] and are
naturally produced by the proteosome [95]. The truncated form of a-syn may serve as a seed to promote cytotoxic aggregation of full length a-syn. Aggregates of truncated C-terminal truncated a-syn will be produced using protocols essentially as described [48, 51, 52]. To generate oligomeric and paired helical filamentous forms of tau, aggregate proteins as described [96].


[0090] Biopanning to isolate nanobodies to the different protein aggregate forms will be performed essentially as described [48, 51, 52, 82, 91]. Nanobody specificity can be determined by a variety of different assays depending on availability of the target antigen.

[0091] a) Antibody Specificity Using ELISA, Western Blot or Dot Blot.

[0092] The binding specificity can be determined by ELISA, western or dot blot, depending on how readily we can purify the target aggregate morphology. The protocols for each of these assays are routinely used in our lab [48, 77-80, 90, 97].

[0093] b) Antibody Specificity Using AFM.

[0094] Nanobody target specificity can be determined using AFM. The height distribution of aggregated particles will increase in size after addition of a nanobody that binds the particles on the mica surface. By monitoring the change in height distribution one can determine target specificity using only minimal amounts of target material [91]. In addition, the target material does not need to be purified since we can monitor changes in selected target heights. The inventors can qualitatively determine specificity by visually identifying which morphologies the nanobody binds to, quantitatively determine the specificity by plotting the change in height distribution data as a function of nanobody concentration as a Langmuir isotherm to calculate K_d values. Alternatively, nanobody specificity for various morphologies can be determined using AFM recognition imaging as described [98].

[0095] Using affinity maturation protocols with a phage display system, the inventors isolated antibodies with over 35-fold better affinity than the original clone using a novel single step selection protocol [108]. The inventors also utilized yeast display libraries to improve the affinity of an scFv specific for Aβ42 over 30-fold from 2.3 × 10^{-10} M to 7.8 × 10^{-10} M.


[0097] Affinity maturation using antibody libraries has routinely improved the affinity of the parent antibody [10]-106, even evolving to obtain femtomolar affinity [109]. The protocols for generating these second generation antibody libraries are described in the various references [10]-106. Basically a second generation library will be constructed essentially as described [110]. Additional library generation can be constructed by varying different CDR regions. While the primers will vary to flank the target CDR sequence, the protocols are essentially the same.

[0098] Based on the results the inventors have obtained with the nanobodies already isolated, the inventors do not anticipate that there will be a need to increase the affinity or stability of the majority of the nanobodies generated. For those clones that do not have sufficient sensitivity in the biosensor assay, the inventors expect that conversion to Fab_2 format will provide sufficient increase in stability and also in affinity due to the bivalent structure. If the Fab_2 format does not have the desired specificity, the individual scFv will be subjected to affinity maturation to increase specificity. After affinity maturation and conversion to Fab_2 format, the antibody fragment does not bind sufficiently well, the antibody will be dropped from further studies.

[0099] Testing the Biosensor ability to detect Aβ, a-syn and tau forms in frozen post mortem samples of CSF taken from healthy, early and late AD, PD and DLB patients: Once the biosensor protocols are developed for sensitive detection of target antigen, and have identified a panel of nanobodies to selected Aβ, a-syn and tau morphologies, for use in the initial test of the biosensor using post-mortem CSF samples from AD, PD, DLB and non-dementia individuals. The samples will be obtained from the Banner/Sun Health Research Institute (BSHRI) Tissue Bank. The inventors will test whether the capturing nanobodies and the EIS arrays have sufficient sensitivity to detect the concentrations of the target species at different stages of these neurodegenerative diseases. For those ligands that cannot be detected, the affinity of the nanobody for the target or the sensitivity of the sensor will be increased as needed. If the target cannot be detected even at femtomolar levels, then we will assume that the requisite signal cannot be detected using the current protocol.

[0100] To test whether the biosensor and nanobody reagents can detect specific protein aggregate species in human CSF samples, the inventors immobilized D5 nanobody, which recognizes an oligomeric a-syn species, to biosensor chips. They then tested five different CSF samples obtained from the BDP (two non-dementia (ND), two PD, and one AD sample) for the presence of this a-syn aggregate species. The two PD CSF samples had the highest concentration of the a-syn aggregated species, the two ND samples had the lowest concentration, and the AD sample was in the middle (FIG. 5). These very promising preliminary results indicate our morphology specific nanobodies can detect target protein species in human CSF samples using the nanomonitor system. They also indicate that levels of specific aggregate protein species have great potential as biomarkers to distinguish different neurodegenerative diseases.

[0101] Methods:

[0102] The BDP currently has CSF available from 147 AD cases with no additional major neuropathologic diagnosis, 46 cases of non-AD dementia and no concurrent diagnosis of AD, 28 non-demented individuals with moderate to severe AD histopathology (Braak III or IV and moderate or frequent CERAD neuritic plaque density) and 29 non-demented individuals with minimal (Braak I or II, zero or sparse CERAD neuritic plaque density) AD histopathology. The non-AD dementia cases are free of significant AD histopathology (CERAD neuritic plaque density zero or sparse; Braak stage I or II) and consist of 18 cases of Parkinson's disease with dementia, 6 cases of vascular dementia, 6 cases of dementia with Lewy bodies, 6 cases of progressive supranuclear palsy, 5 cases of hippocampal sclerosis dementia and 5 cases of dementia lacking distinctive histopathology. In addition, the BDP has brain tissue and CSF from 131 patients with PD, 131 with AD with Lewy Bodies, and 82 with DLB and over 200 elderly controls.


[0104] To determine whether C6 nanobody could also recognize small oligomeric Aβ aggregates in brain tissue, the inventors tested different age mouse brain tissue from wild
type and triple transgenic (3xTg) mice developed by LaFerla et al. (34). Brain extracts from Aβ over-expressing transgenic mice (Tg2576) and control mice were homogenized and run on a 10% Tris-Tricine gel, and transferred onto a nitrocellulose membrane for western blot analysis. The membrane was probed with C6 nanobody using a 9E10-tyramine primary and streptavidin-HRP as secondary antibody and stained with DAB (Sigma). Staining intensity of bands corresponding to 40 kDa was quantified using ImageJ software and compared to the background. Samples with standard deviation a <2 times background are denoted as −; b 2-3 times background as +; and c 3-4 times background as ++ and d >4 times background as ++++. Thus, when the tissues were probed with C6 nanobody, strong reactivity was observed with 22 and 27 weeks old 3xTg samples, while little or no binding was observed with similar aged mice or 68.4 week 3xTg mice (Table 2).

### Table 2

<table>
<thead>
<tr>
<th>Mouse Type</th>
<th>Age</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>22 wks</td>
<td>−</td>
</tr>
<tr>
<td>Wild Type</td>
<td>28.7 wks</td>
<td>−</td>
</tr>
<tr>
<td>Transgenic</td>
<td>10 wks</td>
<td>++</td>
</tr>
<tr>
<td>Transgenic</td>
<td>22 wks</td>
<td>+++</td>
</tr>
<tr>
<td>Transgenic</td>
<td>27.3 wks</td>
<td>+++</td>
</tr>
<tr>
<td>Transgenic</td>
<td>68.4 wks</td>
<td>+</td>
</tr>
</tbody>
</table>

Next, the C6 antibody fragment was reacted with blotted aliquots of soluble homogenized samples obtained from healthy (ND) or AD human brain tissue. Brain extracts from the medial temporal gyms of Non Diseased patients (ND) and Alzheimer’s disease patients (AD) were homogenized and deposited onto a nitrocellulose membrane and probed with C6 nanobody. Staining intensity of the dot blot was quantified using ImageJ software and compared to the background. Samples with standard deviation a <2 times background are denoted as −; b 2-3 times background as +; and c 3-4 times background as ++ and d >4 times background as ++++. Thus, C6 reacted strongly with brain tissue from AD patients who had moderate plaque frequency, but showed little or no reaction with brain tissue from AD patients with severe plaques or with the ND patients (Table 3).

### Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample Description</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND1</td>
<td>No plaque</td>
<td>−</td>
</tr>
<tr>
<td>ND2</td>
<td>No plaque</td>
<td>−</td>
</tr>
<tr>
<td>ND3</td>
<td>Moderate Frequency Plaque</td>
<td>+</td>
</tr>
<tr>
<td>ND4</td>
<td>Moderate Frequency Plaque</td>
<td>−</td>
</tr>
<tr>
<td>ND5</td>
<td>Moderate Frequency Plaque</td>
<td>−</td>
</tr>
<tr>
<td>ND6</td>
<td>Moderate Frequency Plaque</td>
<td>+</td>
</tr>
<tr>
<td>AD1</td>
<td>Moderate Frequency Plaque</td>
<td>++</td>
</tr>
<tr>
<td>AD2</td>
<td>Moderate Frequency Plaque</td>
<td>+++</td>
</tr>
<tr>
<td>AD3</td>
<td>Moderate Frequency Plaque</td>
<td>++</td>
</tr>
<tr>
<td>AD4</td>
<td>Severe Plaques</td>
<td>+</td>
</tr>
<tr>
<td>AD5</td>
<td>Severe Plaques</td>
<td>−</td>
</tr>
<tr>
<td>AD6</td>
<td>Severe Plaques</td>
<td>−</td>
</tr>
</tbody>
</table>

Preliminary results were obtained with five postmortem CSF samples using our nanomonitor sensor with immobilized D5 nanobody which recognizes an oligomeric a-syn species [2]. The inventors have now completed testing on nine different CSF samples obtained from the BSHRI HDP (three non-demented (ND), three PD, and three AD samples) for reactivity with D5 and also have data with these same nine samples using the A4 nanobody which recognizes an oligomeric form of Aβ [4]. The biosensor is sufficiently sensitive that the CSF samples can be diluted 10 fold and the specific oligomeric species are readily detected. Using the D5 anti-a-syn nanobody, the three ND CSF samples (ND1-3) all showed consistently low, non-zero readings, the three AD samples (AD1-3) had substantially higher values, and two of the three PD samples (PD1, PD2) had the highest values, while the third PD sample (PD3) had a reading similar to the ND samples (FIG. 6A). The pathology associated with the third PD sample (PD3) indicates that the patient had Parkinsonism caused by Multiple System Atrophy (MSA) and was not a true PD case. The presence of aggregated a-syn in AD brain tissue has been documented[5], and here it is shown that aggregated a-syn is also detected in CSF from human AD patients, but at slightly lower levels than in PD cases. Using the A4 anti-Aβ nanobody, the three ND samples (ND1-3) and three PD samples (PD1-3) did not show any binding to A4, while the three AD samples (AD1-3) all showed significant presence of this oligomeric Aβ species (FIG. 6B). Therefore using only a positive or negative binding result based on a threshold value of ND samples, one can readily distinguish between ND, PD and AD samples using only two of the morphology specific reagents (Table 4). It is expected that evaluating concentrations of the different oligomeric species will provide additional information about disease progression and can be very valuable as a tool to assess effectiveness of different therapeutic strategies.

### Table 4

<table>
<thead>
<tr>
<th>Antibody</th>
<th>ND1</th>
<th>ND2</th>
<th>ND3</th>
<th>PD1</th>
<th>PD2</th>
<th>PD2</th>
<th>AD1</th>
<th>AD2</th>
<th>AD3</th>
</tr>
</thead>
<tbody>
<tr>
<td>D5</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A4</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The additional data presented herein provide further evidence that different oligomeric protein forms, including Aβ species, exist in human tissue and in cell and animal models of AD, and that our reagents can specifically recognize these species, that they can be used to differentiate different stages of AD and to distinguish between disease samples. It is also shown that one can accurately and sensitively detect target protein species directly from human CSF samples using the electronic biosensor system. Using just two nanobodies from our proposed panel of morphology specific reagents, we are able to very clearly differentiate ND, PD and AD CSF samples. Our reagents not only distinguish between different neurodegenerative diseases but potentially between different causes of these diseases. These results provide very encouraging evidence that levels of specific aggregate protein species have great potential as biomarkers to distinguish between different neurodegenerative diseases, to characterize progression of these diseases, and to monitor the effectiveness of different therapeutic strategies.
The following material provides additional data showing morphology specific nanobodies specifically recognize different naturally occurring Aβ aggregates that are diagnostic of different stages of Alzheimer’s disease and data showing that the nanomonitor biosensor in conjunction with the nanobodies can readily and sensitively detect specific morphologies of target antigens in CSF samples. The results indicate that detection of specific toxic protein species in CSF has great potential as a diagnostic for neurodegenerative diseases.

Table 5 summarizes additional data for tests using CSF samples. In the table, (+) indicates reactivity of the sample with the target antibody, (−) indicates no reactivity, (+?) indicates that the testing is not completed yet, but the ones evaluated so far are positive, (−/+?) means some are positive and some are negative, and (?) means that the experiments are not yet completed. The data is very encouraging since with only four of the nanobodies, the inventors are able distinguish between non-demented (ND), Parkinson’s (PD), Alzheimer’s (AD) and Dementia with Lewy Bodies (DLB).

<table>
<thead>
<tr>
<th>Antibody Fragment</th>
<th>Library source</th>
<th>Specificity</th>
<th>Assays to validate</th>
<th>Applications demonstrated</th>
<th>SEQ ID NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>Tomlinson (MRC)</td>
<td>Oligomeric Abeta 3-day aggregates</td>
<td>Dot blot, ELISA, AFM (Note: 3-day oligomeric target is not stable for westerns)</td>
<td>Human AD brain tissue, Human CSF, Mouse AD brain tissue, (Dot blot assays), Immunohistochemistry</td>
<td>amino acid SEQ ID NO: 2; nucleic acid SEQ ID NO: 8</td>
</tr>
<tr>
<td>E1</td>
<td>Tomlinson (MRC)</td>
<td>Oligomeric Abeta 1-day aggregates</td>
<td>Dot blot, ELISA, AFM (Note: 1-day oligomeric target is not stable for westerns)</td>
<td>Human AD brain tissue, Human CSF, Mouse AD brain tissue, (Dot blot assays)</td>
<td>amino acid SEQ ID NO: 3; nucleic acid SEQ ID NO: 9</td>
</tr>
<tr>
<td>C6</td>
<td>Sheets (UCSF)</td>
<td>Oligomeric Abeta brain derived</td>
<td>AFM</td>
<td>Human AD brain tissue, Human CSF, Mouse AD brain tissue, (Dot blot assays)</td>
<td>amino acid SEQ ID NO: 1; nucleic acid SEQ ID NO: 10 and amino acid SEQ ID NO: 15</td>
</tr>
<tr>
<td>D5</td>
<td>Tomlinson (MRC)</td>
<td>Oligomeric α-synuclein 3-day aggregates</td>
<td>Dot blot, time course, ELISA, AFM, western blot analysis</td>
<td>Human PD brain tissue, Human CSF, Mouse PD brain tissue, (Dot blot assays), Western blot, Immunohistochemistry</td>
<td>amino acid SEQ ID NO: 4; nucleic acid SEQ ID NO: 11</td>
</tr>
<tr>
<td>10F1</td>
<td>Tomlinson (MRC)</td>
<td>Oligomeric α-synuclein 7-day aggregates</td>
<td>Dot blot, time course, ELISA, AFM, western blot analysis</td>
<td>Human PD brain tissue, Mouse PD brain tissue, (Dot blot assays), Western blot, Immunohistochemistry with tissue and cells</td>
<td>amino acid SEQ ID NO: 5; nucleic acid SEQ ID NO: 12</td>
</tr>
<tr>
<td>E6</td>
<td>Tomlinson (MRC)</td>
<td>Fibrillar aggregates (likely not protein specific)</td>
<td>Dot blot, time course, ELISA, AFM</td>
<td>Human PD brain tissue, Human CSF, Mouse PD brain tissue, (Dot blot assays), Western blot, Immunohistochemistry with tissue and cells</td>
<td>amino acid SEQ ID NO: 6; nucleic acid SEQ ID NO: 13</td>
</tr>
</tbody>
</table>
[0112] In certain embodiments, the C6 nanobody has a sequence of SEQ ID NO:1:

```
EXPIAYGSKIVITREDAGHSGKDAGVDIQLSLPLCAASGFTFSSYAVKLEAVQSGSLRLSCAASGFTFSSYAMSTHPEL
```

[0113] In certain embodiments, the C6 nanobody lacks the initial 1-15 amino acids of SEQ ID NO:1.

**Example 2**

**[0114]** 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 beta-amyloid (Aβ) and α-synuclein (α-syn), can be an effective diagnostic assay for these diseases. CSF levels of tau and Aβ 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 Aβ and α-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 neurodegenerate disease.

**[0115]** 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. Diagnosis of these diseases is challenging as other neurodegenerative diseases such as Lewy are often classified as having Mild-Cognitive Impairment (MCI), a term describes early, non-disabling cognitive disorders.

**[0116]** Although MCI describes a transitional state between normal Body Dementia (LBD), frontotemporal dementia and vascular dementia may share similar symptoms, but have different mechanisms and pathology. Patients in early stages of dementia aging and dementia, not all MCI cases progress to dementia. Pathological changes associated with these different dementia have been shown to occur long before symptoms are evident, 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.

**[0117]** Numerous studies have looked at key biomarkers for diagnosing neurodegenerative diseases, especially AD where levels of tau, phosphorylated tau and amyloid-beta 42 (Aβ42) 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. These very promising studies indicate that CSF biomarkers can be a valuable tool for both facilitation of diagnosis of neurodegenerative disease and to assess effectiveness of different therapeutic strategies.

**[0118]** While CSF protein biomarkers such as tau and Aβ 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 (Aβ) with AD, alpha-synuclein (α-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 Aβ accumulation and plaque formation as being central to the pro-
gression of AD, however, while the presence of amyloid plaques does not correlate well with the progression of AD, the presence of various different soluble Aβ species does. Similarly, soluble aggregate forms of tau have been correlated with AD and soluble forms of a-syn have also been correlated with PD.

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 Aβ 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 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 (Barkhordarian et al., Isolating recombinant antibodies against specific protein morphologies using atomic force microscopy and phage display technologies. Protein Eng Des Sel 2006, 19, (11), 497-502; Zameer et al., Anti-oligomeric Aβeta single-chain variable domain antibody blocks Aβeta-induced toxicity against human neuroblastoma cells. J Mol Biol 2008, 384, (4), 917-28; et al., Isolation of a human single chain antibody fragment against oligomeric alpha-synuclein that inhibits aggregation and prevents alpha-synuclein-induced toxicity. J Mol Biol 2007, 368, (4), 1132-44; Emadi et al., Detecting morphologically distinct oligomeric forms of alpha-synuclein. J Biol Chem 2009, 284, (17), 11048-58.) These morphology specific reagents are ideal candidates to determine whether specific aggregate species of Aβ and a-syn can be detected in CSF and whether they have potential as markers to help study and diagnose neurodegenerative diseases. We have 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 (Bothawut et al., Nanomonitor: electrical immunoassay for protein biomarker profiling. Nanomed 2008, 3, (4), 423-36; Reddy et al., Nanomonitor: Protein biosensors for rapid analyte analysis. Ieee Sensors Journal 2008, 8, (5-6), 720-723; Venkatraman et al., Iridium oxide nanomonitor: Clinical diagnostic devices for health monitoring systems. Biosensors & Bioelectronics 2009, 24, (10), 3079-3083). 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. 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 Aβ and a-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
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 silicon 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 μm in width, 5 mm in length and 800 nm in thickness with rounded edges to minimize fringe effects during the application of a sinusoidal voltage input signal (FIG. 9A).

A nanoporous alumina membrane is soldered onto the interdigitated 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. 9B).

Finally, a circular, silicon micro 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. 9C).

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 Helmoltz–Gouy–Chapman model with Simes correction (Chang, B.Y., Park, S.M., Electrochemical impedance spectroscopy. Annu Rev Anal Chem (Palo Alto...
Calif.) 3, 207-29; Lisdat, F., Schafer, D., The use of electrochemical impedance spectroscopy for biosensing. Anal Bioanal Chem 2008, 391, (5), 1555-67. Since binding of antigens to the nanobodies is free of any bio-chemical mediators, the impedance changes within the electrical double layer (FIG. 9D) is non-Faradic, 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 (Bothara, Met. Nanomoni- tors: electrical immunosassays for protein biomarker profil- ing. Nanomed 2008, 3, (4), 423-56). For probing the imped- ance 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 (Ganry Industries Reference 600 potentiostat).

[0128] Another commonly used electrochemical immuno- sensing technique is the pulsed amperometry. This method involves the immobilization of an immune-reactant compo- nent on the electrode transducer and the use of an electro- chemical active substance produced by enzymatic reaction for signal generation (Lei, C. X., F. C. Gong, et al., Amperometric immunosensor for Schistosoma japonicum antigen using antibodies loaded on a nano-Au monolayer modified chitosan-entrapped carbon paste electrode. Sensors and Actuators B: Chemical 2003, 96(3): 582-588). 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 oxidizing agent, redox active interferences which either react at the electrode and/or couple with the reagent couple, and inadequate temperature control (Kissinger, P. T., Introduction to Amperometric Biosensor Configuration. CURRENTSEPA- RATIONS.COM and Drug Development 1997, 16(3)). The EIS technique eliminates most of these problems since it doesn’t rely on the redox properties of the analyte and does not need an enzyme inhibitor. Another fundamental differ- ence between the two techniques is the sensing mechanism. Amperometry involves detection of ions in the solution by applying voltages on the 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.

[0129] Nanobody Immobilization to Sensor Surface

[0130] Nanobodies are immobilized onto the electrode sensor surface using a chemical linker. The electrode surface is first amine functionalized using 3-Aminopropyl Triethoxy- silane (APTES, 2% in acetone buffer, Thermo Scientific Inc.). A 100 µl aliquot of 2% APTES is applied on the electrode surface and incubated at room temperature for 30 seconds. Excess APTES was then removed by flowing acetone over the surface. The aluminia 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) is used to cross link the nanobodies to the electrode surface. The DSP (thiol linker) is added for 30 minutes to allow conjugation to the silanized electrode surfaces which form the base of the nanowell. After conjugation of the linker to the nanowell surfaces (primarily to the base of the nanowell), a 40 µl aliquot of the nanobody (1 mg/ml) is added for 15 minutes, followed by addition of 40 µl of Bovine Serum Albumin (BSA) (2 mg/ml) 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.

[0131] Preparation of CSF Samples.

[0132] Post mortem CSF Samples from AD, PD, MSA and non-diseased autopsy confirmed sources were generously provided by Dr. Thomas Beach (Civin Laboratory for Neu- ropathology, Banner/Sun Health Research Institute, Sun City, Ariz.). The post-mortem interval for collection of samples is less than three hours. Samples were diluted to one-tenth of their original concentration in 1xPBS buffer and frozen.

[0133] Expression and Purification of Nanobodies.

[0134] D10, D5 and A4 nanobodies were produced and purified essentially as previously described (Zameer et al., Anti-oligomeric Abeta single-chain variable domain antibody blocks Abeta-induced toxicity against human neuroblastoma cells. J Mol Biol 2008, 384, (4), 917-28; Emadhi et al., Detecting morphologically distinct oligomeric forms of alpha-synuclein. J Biol Chem 2009, 284, (17), 11048-58). D10 was used to calibrate the sensor chip since it binds to all forms of a-syn including monomeric facilitating preparation of a-syn standards of known concentration. The D5 nanobody specifically recognizes a small oligomeric a-syn species (Emadhi et al., Isolation of a human single chain antibody fragment against oligomeric alpha-synuclein that inhibits aggregation and prevents alpha-synuclein-induced toxicity. J Mol Biol 2007, 368, (4), 1132-44), while A4 specifically recognizes a small oligomeric Aβ species (Zameer et al., Anti-oligomeric Abeta single-chain variable domain antibody blocks Abeta-induced toxicity against human neuroblastoma cells. J Mol Biol 2008, 384, (4), 917-28). Nanobody specificity was demonstrated by several different assays including time course dot blot, ELISA, western blot and Atomic force microscopy based assays (Zameer et al., Anti-oligomeric Abeta single-chain variable domain antibody blocks Abeta-induced toxicity against human neuroblastoma cells. J Mol Biol 2008, 384, (4), 917-28; Emadhi et al., Isolation of a human single chain antibody fragment against oligomeric alpha-synuclein that inhibits aggregation and prevents alpha-synuclein-induced toxicity. J Mol Biol 2007, 368, (4), 1132-44; Wang et al., Characterizing Antibody Specificity to Different Protein Morphologies by AFM. Langmuir 2008. The supernatant and cell lysate from a I L 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 (Zameer et al., Anti-oligomeric Abeta single-chain variable domain antibody blocks Abeta-induced toxicity against human neuroblastoma cells. J Mol Biol 2008, 384, (4), 917-28). 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, Calif.) and western blotting, and the concentration was determined using biocinchonic acid (BCA) protein assay (Pierce, Ill.).


[0136] A-syn was prepared and purified in our lab as previously described (Emadhi et al., Isolation of a human single chain antibody fragment against oligomeric alpha-synuclein that inhibits aggregation and prevents alpha-synuclein-induced toxicity. J Mol Biol 2007, 368, (4), 1132-44; Volles et
al., Relationships between the sequence of alpha-synuclein and its membrane affinity, fibrilization propensity, and yeast toxicity. J Mol Biol 2007, 366, (5), 1510-22. 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 minutes, 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 Aβ 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 (Zhou, C.; Emadi, S.; Sierks, M. R.; Messer, A.), a human single-chain Fv intrabody blocks aberrant cellular effects of overexpressed alpha-synuclein. Mol Ther 2004, 10, (6), 1023-31), 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 (Botham et al., Nanomonitor: electrical immunosassays for protein biomarker profiling. Nanomed 2008, 3, (4), 423-36; Reddy et al., Nanomonitor: Protein biosensors for rapid analyte analysis. IEEE Sensors Journal 2008, 8, (5-6), 720-723), 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 to 1 μg/ml) to immobilized D10 using a frequency range from 50 Hz to 1 MHz. A frequency of 100 Hz gave maximum visible shifts in the impedance induced by biomolecule binding (FIG. 10).

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 picogram/ml (FIG. 11) 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, a-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. 11). The nanobody also specifically reacts with the a-syn target since no signal is observed with the control protein antigen, BSA (FIG. 11).

After demonstrating that we can detect femtomolar concentrations of a-syn target using immobilized nanobodies, we next determined whether we could detect specific morphologies of a-syn in post-mortem CSF samples. While we utilized the D10 nanobody, which recognizes all forms of a-syn, for the calibration studies since we could accurately measure the concentration of monomeric a-syn present, in order to detect specific oligomeric forms of a-syn and Aβ in CSF samples, we next utilized two other nanobodies which selectively recognize either a specific oligomeric form of a-syn (D5) or of Aβ (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 7. 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. 12A) and A4 (FIG. 12B).

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[0141] 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.

[0145] Oligomeric species of both a-syn and Aβ are readily detected in post-mortem CSF samples even when assayed at 10-fold dilution (FIG. 12A, B). The results indicate that the
levels of oligomeric α-syn and Aβ species in CSF varies depending on the disease, and that these oligomeric species have great promise as biomarkers for distinguishing between different neurodegenerative diseases.

[0146] Discussion

[0147] CSF levels of Aβ, tau, and phosphorylated tau were shown to have promise as biomarkers for diagnosing AD. However, increasing evidence indicates that various soluble aggregated oligomeric forms of Aβ, 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 Aβ 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.

[0148] A rapidly growing body of evidence indicates that oligomeric forms of Aβ are key factors in the onset and progression of AD. Aβ forms a number of soluble intermediates or metastable structures which can contribute to toxicity. Cortical levels of soluble Aβ correlate well with the cognitive impairment and loss of synaptic function. Small, soluble spherical or annular aggregates of Aβ were shown to be neurotoxic, and oligomeric forms of Aβ, created in vitro or derived from cell cultures inhibit long term potentiation (LTP). The concentration of oligomeric forms of Aβ are elevated in transgenic mouse models of AD and human AD brain and CSF samples and the presence of an SDS stable dimeric form of Aβ associates well with dementia in AD patients. Disruption of neural connections near Aβ plaques was also attributed to oligomeric Aβ species, a halo of oligomeric Aβ surrounds Aβ plaques causing synaptic loss, and oligomeric Aβ was shown to disrupt cognitive function in transgenic animal models of AD. Different size oligomers of Aβ have been correlated with AD, including a 56 kD aggregate and smaller dimeric, trimeric and tetrameric forms. Therefore specific detection of soluble oligomeric Aβ species holds great promise as a biomarker for studying the progression of AD.

[0149] Similarly, formation of oligomeric aggregates of α-syn has also been correlated with PD and other synucleinopathies. α-syn is a major component of Lewy bodies and neurites. While wild-type and mutant forms of α-syn associated with familial cases of PD, A30P, E46K and A53T, can assemble into Lewy body like fibrils in vitro, the mutations increase the total rate of oligomerization compared to the wild-type form of α-syn. Different morphologies of α-syn have different affinities for various membranes, and both oligomeric and fibrillar forms have been shown to disrupt membrane permeability and integrity. Aggregated forms of α-syn were shown to induce toxicity in dopaminergic neurons in vivo and several different oligomeric morphologies were shown to have different toxic mechanisms and effects on cells. Oligomeric forms of α-syn were shown to be toxic to neuronal cells, and toxic oligomeric α-syn forms were identified in living cells, in human plasma from PD patients, and in human PD brain tissue. Elevated levels of soluble oligomeric α-syn species were also detected in post-mortem brain extracts from patients with LBD, even though monomeric α-syn levels in CSF were not able to discriminate between LBD and AD. Therefore the presence of various oligomeric α-syn species in CSF is also a very promising biomarker for studying the progression of various neurodegenerative diseases.

[0150] We developed a novel biosensor for sensitive detection of biomolecules from clinical samples and show here that the Nannonitor 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, and have isolated nanobodies that bind two different oligomeric α-syn species and two different oligomeric Aβ species. The different oligomeric specific nanobodies do not show cross-reactivity, so the nanobodies binding oligomeric α-syn do not bind oligomeric Aβ 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. Therefore these nanobodies represent excellent tools to detect specific oligomeric aggregates of Aβ and α-syn in both clinical and animal model samples.

[0151] Here we show that detection of an oligomeric Aβ species in CSF samples using the A4 nanobody very sensitively distinguishes the AD CSF samples from the PD and ND samples (FIG. 12B). The oligomeric α-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. 12A). Interestingly, the α-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. 12C). 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 α-syn and Aβ.

CONCLUSION

[0152] 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 Aβ, α-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 Aβ and tau species can correctly predict progression to AD validating this concept. Since increasing evidence indicates that small soluble oligomeric forms of proteins including Aβ and α-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.

All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms, meaning “including, but not limited to”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

LITERATURE CITED


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Lys Asp Ser Ala Ser Phe Asp Tyr Trp Gln Gln Gly Thr Leu Val Thr
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**LOCATION:** (715)-(718)
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agcagctac ccctcagctg gtcctcgac cgctcgggga agggggctga gttgggtctca 190
gcaggcagc atacgttgctgc gcgcacaact tgcagcagat ccgtaagagg cgcgttgtcac 240
atccggcg acaattccaa gacacgcttg tattctgcaaa tgcagcagtt gagggagcag 300
gacagcagcc tacattactct tgtcgacgcgt tgtcactactc ggccagggga 360
acgcgtgtca ccggcagcg ccggcagggc gttccgggac gcgtgggga ggtttggcccgc 420
gggttcagc gcacgacagt gccacgtct ccaccccctcg tgtcgtgacct tgtgcagcag 480
agagttccac tcaactgtgc gcacagacagt gcacatgact gcatttttaat tgttgatcag 540
cagacacacag ggaacagctgg ttaacctctcg actctctctctc ccacattttggg 600
gtctccacag ggatttgggtagttgatc tggcagagtttt ctcacccacatttgcagctgg 660
cgcaaatctct tactactactg tcgactacag gcggagactgg gcctttccccct 720
tcgcaactctt acagatttttct caactatcact tcgactacag gcggagactgg gcctttccccct 780
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gecaagcgag catagagctgg ttcggccagg cctccaggaaa ggggtcctag tgggctctcat 180
tcatccgacg tggaggttagg gggacagctg agctagactc ctgcaggggc cgggtcaccuu 240
tctccagaga cattctcaag aacacgctgt aatcctacat gacacgcttg agagccgagg 300
acagccgctg atattaactct gggaaaccgc ccagagagtt tgaactactgg gcacagggaa 360
cctggtctac gctctctgcag gttggaggcc gttacagggc aggctcggca ggcggctggag 420
gggacgaggg cttctctatag accagctctc cctctctctct gctctctctct gtatggagaa 480
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aaaatgtttta anacacacac atccacaaaa ttctattt 877

<210> SEQ ID NO 10
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<212> TYPE: DNA
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cagctgaggg cttctctatag accagctctc cctctctctct gctctctctct gtatggagaa 180
agacgaccc ggaagccgact atccgaaa aggacacgg aacaccgaa tgggccc gaggtctgtg 240
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360
gctctctcta gtaagagggc gttaaggggtc agtgggtact ggggttgccg gataggaat parted

420
ttggtgtcag ctgtccccgc actctcggcc tgggtctctgt ggcacagggg caccatac parted

480
cctgcaagctc gagccagagtt ttagttcaaa ctcaacaacta aagaactact tagctttgta parted

540
cacagcaaaaa ccaagacactb tcaagtaagtt tcaaggataaa cccggaact parted

600
cggggtctctt gacggattca gttgacgagc gtttggaaca gaaatctcct aatactcag parted

660
cagctggcag gatggatgt tgcagttta ttagcctcag ccaatttata tgaactctc parted

720
gacttttgccc cggggaacca agcttgagat ccaacagttg gcgcacaagc attacaac parted

780
tcaaggggccc gcaagaaacaa aacatcactac aagagagac parted

821

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

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120
gccagggctt aggggacgggg tgtggggtcgt tgtagttgga ttagttcattag parted

180
cagactgcg acgctgggtg aagggcggttg ttagttcattag parted

240
cagctgccg acgctgggtg aagggcggttg parted

300
cacatggccga ggtgacgtctg tgtggaatcg ggggggtccc parted

360
tgagaccttc ctgtgtgacgc tctggactcaa cccttagcgct ttagggcgca parted

420
gccagggctt aggggacgggg tgtggggtcgt parted

480
gccagggctt aggggacgggg parted

540
tgagaccttc ctgtgtgacgc parted

600
gccagggctt aggggacgggg parted

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tgagaccttc ctgtgtgacgc parted

780
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822

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120
agcagctctccctgtccagctctgtgctctcctagagctgctctgcctggagcttggccttgccctcttc
180
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660
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720
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780
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843
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agcgatcg cctgagctg gtctggcagc gtctgggaga gggtgcggga gtggctcga 180
tattagctc ggtggtggtg tactataaat tgtgcagact cctggagagg cgggttcacc 240
atctcagag acacctcaca gaacagcgtg tattctgcaa tgaacagcct gaggacgcag 300
gacaagcggc tgttactaag ttgcaagaatt tcgttcgttt ttgactactg ggcaagcggg 360
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agagtcacca tcgtgagcgc gcagacgctag acatcaggca gcattattaaa ttcgtatcag 540
cggacacggc gcagacggcc ttaacctctggt acatctgcct gcccctatttt gccaatggygg 600
gtccacacta ggcggcagct caggctgtct gggagtctga acatcctca acacgctg 660
cacggctctt acagagttgg aatctacac tgctgaagag cggcttcata gcgcctacg 720
tgcggcag gcagagcagtc gcagatcaca cggcagctgc cagagacagtc tccttctac 780
gggnccnnna naacaacaact cttcctccaat ctaaatctt ggtgggacnn gcaatctgt 840
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cacggccgac gcagctgggtt gcgttactagc atcaagtctctg cgggtcgctctg 180
tactatgcag acatcggcat gggctggctgg cagctctcaca gacaactctg cagaggtcag 240
tcgtatcctcg cttgagctgg aagcgtgctg cggctgttcct gcgtggtggc 300
attagggca agttggttca atgggaatcgt gcggctgttg ccgggtttctg gggcaggtgg 360
cccccccccac gcggctgtgttg ttcggttcag cagatcagtc aatcctctgcctctttctcct 420
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agctatattaa atggtatca gcagaaacca gggaagccc ctaagctcct gatctatgtct 540
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ttcctctca ccatcagcag tctgcaacct ggagatgttg caacctacta ctgcacagcag 660
agttcagta cccgacgtt cgggaaggg accaagctgg aaataacaagc tgccgcccagca 720
catcatcac accatcaacg ggcgccgaga caaaaactca ttcacagaga gatct 776

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1  5  10  15
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20  25  30
Ser Tyr Ala Met Ser Trp Val Arg Glu Ala Pro Gly Lys Gly Leu Glu
35  40  45
Trp Val Ser Ala Ile Ser Gly Ser Gly Ser Thr Tyr Tyr Ala Asp
50  55  60
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr
65  70  75  80
Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr
85  90  95
Tyr Cys Ala Lys Ser Tyr Gly Ser Val Lys Ile Ser Cys Phe Asp Tyr
100 105 110
Trp Gly Glu Ser Thr Leu Val Thr Val Ser Ser Gly Gly Gly Gly Ser
115 120 125
135 140
Ser Pro Asp Ser Leu Ala Val Ser Leu Gly Arg Ala Thr Ile Asn
145 150 155 160
Cys Lys Ser Ser Gln Ser Val Leu Tyr Asn Ser Asn Lys Asn Tyr
165 170 175
Leu Ala Trp Tyr Glu Glu Ser Pro Gly Gin Ser Pro Glu Leu Leu Ile
180 185 190
Tyr Thr Ala Ser Thr Arg Glu Ser Gly Val Pro Asp Arg Phe Ser Gly
195 200 205
Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala
205 210 215 220
Glu Asp Val Ala Val Tyr Cys Glu Gin Phe Tyr Ser Thr Pro Pro
220 225 230 235 240
Thr Phe Gly Gin Gly Thr Leu Glu Ile Lys Arg Ala Ala Ala His
245 250 255
His His His His His Gly Ala Ala Glu Gin Lys Leu Ile Ser Glu Glu
260 265 270
Asp Leu Asn Gly Ala Ala
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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic peptide

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1  5

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Ala Ile Ser Gly Ser Gly Gly Ser Thr Tyr Tyr Ala Asp Ser Val Lys
1  5  10  15

Gly

<210> SEQ ID NO 18
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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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1  5  10

<210> SEQ ID NO 19
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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic peptide

<400> SEQUENCE: 19
Lys Ser Ser Glu Ser Val Leu Tyr Asn Ser Asn Lys Asn Tyr Leu
1  5  10  15

Ala

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<400> SEQUENCE: 26

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<210> SEQ ID NO 27
1. A biosensor comprising:
   a. a microelectrode array base platform containing an array of conductive-material sensing sites, wherein each sensing site is comprised of a working electrode (WE) and a counter electrode (CE) wherein the surface area ratio of CE:WE is between about 20:1 and 300:1;
   b. a nanoporous membrane overlaid on the platform wherein the membrane forms nanowells, wherein each nanowell comprises immobilized therein an antibody agent that specifically detects a marker of a neurodegenerative disease;
   c. a microfluidic encapsulant to enable lateral flow of reagents over and into the nanowells.
2. The biosensor of claim 1, wherein the conductive material is platinum, gold, silver, or copper.
3. -6. (canceled)
7. The biosensor of claim 1, wherein the nanoporous membrane is aluminum.
8. The biosensor of claim 1, wherein the nanowell has an effective diameter of about 10 nm to 500 nm.
9. (canceled)
10. The biosensor of claim 1, further comprising a readout amplifier electrically connected to nano-pores in one or more regions of the membrane.
11. The biosensor of claim 1, further comprising a fluid chamber.
12. (canceled)
13. The biosensor of claim 1, wherein the base platform is an insulator or includes an insulator portion.
14-15. (canceled)
16. The biosensor of claim 1, further comprising one or more conductor strips coupling the biosensor to a sensor, wherein the sensor is coupled to a multiplexer.
17. (canceled)
18. The biosensor of claim 1, wherein the marker of a neurodegenerative disease is an Aβ, a-syn or tau morphology.
19. The biosensor of claim 1, wherein the antibody agent is an antibody, an Fab₂, or a nanobody.
20. The biosensor of claim 19, wherein the nanobody is a C6, A4, E1, D5, 10H, 6E, D10 or BSEC1 nanobody.
21. (canceled)
22. A method of detecting the presence of a neurodegenerative disease comprising contacting a physiological sample from a subject suspected of having a neurodegenerative disease with a biosensor of claim 1 and measuring the output impedance of the biosensor in the presence of the biological fluid wherein a change in the measured output impedance across a sensing site is indicative of the presence of a marker of a neurodegenerative disease.
23-29. (canceled)
30. A biosensor device comprising:
   (a) a printed circuit board platform,
   (b) a nanoporous membrane, and
   (c) a micro fluidic chamber.
31. The biosensor device of claim 30, wherein the printed circuit board platform comprises inter-digitated working and counter electrodes.
32. The biosensor device of claim 31, wherein the electrodes are tin oxide, gold, platinum, silver, or copper, or a combination of these materials.
33. (canceled)
34. The biosensor device of claim 30, wherein electrodes are about 600-1000 μm in width, about 2-10 mm in length and about 600-1000 nm in thickness.
35. (canceled)
36. The biosensor device of claim 31, wherein the nanoporous membrane is contacted onto the interdigitated electrodes.
37. (canceled)
38. The biosensor device of claim 30, wherein the nanoporous membrane comprises alumina, polycarbonate, a metal oxide, or a ceramic-based material.
39. (canceled)
40. The biosensor device of claim 30, wherein the nanoporous membrane is 100-500 nm thick, has a lateral diameter of 5-20 mm with pore diameters of 100-500 nm.
41. (canceled)
42. The biosensor device of claim 30, wherein the nanoporous membrane has a porosity of about 25% to 50%.
43. The biosensor device of claim 30, wherein the micro fluidic chamber is silicone, acrylic, plastic, or any other bio-compatible hydrophilic material.
44. (canceled)
45. The biosensor device of claim 30, wherein the micro fluidic chamber forms an enclosure for the printed circuit board platform and the nanoporous membrane to prevent evaporation.
46. The biosensor device of claim 45, wherein the micro fluidic chamber has a volume of about 10 μl to about 5 ml.
47. (canceled)
48. The biosensor device of claim 31, wherein the electrodes are contacted with a negatively-charged substance to form a coated electrode surface.
49. The biosensor device of claim 31, wherein the electrodes are contacted with an amine or BST.
50. (canceled)
51. The biosensor device of claim 31, further comprising an antibody agent immobilized onto the printed circuit board platform or the coated electrode surface.
52. The biosensor device of claim 51, wherein the antibody agent is an antibody, an Fab₂, or a nanobody.
53. The biosensor device of claim 52, wherein the nanobody is a C6, A4, E1, D5, 10H, 6E, D10 or BSEC1 nanobody.
54-57. (canceled)