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(54) Title: DIAGNOSIS AND TREATMENT OF NEURODEGENERATIVE DISORDERS

(57) Abstract: The invention relates to the diagnosis and treatment of neurodegenerative disorders, such as Parkinson' and Alzheimer's disease. In particular, the invention relates to methods for assessing whether an individual has a neurodegenerative disorders before clinical symptoms appear. The invention also relates to methods for delaying or preventing the onset of clinical symptoms associated with neurodegenerative disorders, as well as to methods for identifying agents useful in the treatment or prevention of neurodegenerative disorders.

Diagnosis and Treatment of Neurodegenerative Disorders

Field of the Invention

The present invention relates to the diagnosis and treatment of neurodegenerative disorders, such as Parkinson' and Alzheimer's disease. In particular, the present invention relates to methods for assessing whether an individual has a neurodegenerative disorders before clinical symptoms appear. The invention also relates to methods for delaying or preventing the onset of clinical symptoms associated with neurodegenerative disorders, as well as to methods for identifying agents useful in the treatment or prevention of neurodegenerative disorders.

Background to the invention

Many neurodegenerative diseases share a common process of misfolding of a native monomeric protein into intermediate oligomeric structures that share a common structural motif, the extended β-sheet, and ultimately into insoluble fibrils, which deposit in the brain leading to progressive neuronal death. Alzheimer's Disease (AD) and Parkinson's Disease (PD) are the most prevalent of these diseases, affecting up to 3% of people over the age of 65. This pathological process starts many years prior to presentation, with the result that significant neuronal loss has already occurred at the onset of clinical symptoms. Thus any disease modifying treatment demands a robust and early diagnostic test that ideally reflects the fundamental pathogenic process underlying neurodegeneration and can be applied before clinical symptoms appear. The relevant protein thought to be associated with disease in PD patients is alpha-synuclein, while in the case of AD it is amyloid-beta and tau.

Alpha-synuclein (α-synuclein; αS) is a small protein expressed at high levels in neuronal cells (Jakes *et al.*, 1994). The central role of alpha-synuclein in the pathogenesis of PD is well established by genetic and pathological data. Point mutations and multiplications of the SNCA gene are sufficient to cause an autosomal dominant form of PD, suggesting that dysfunction of the alpha-synuclein protein is a primary step in disease pathogenesis (Polymeropoulos *et al.*, 1997; Krüger *et al.*, 1998; Zarranz *et al.*, 2004; Singleton *et al.*, 2003; Chartier-Harlin *et al.*, 2004). In addition to this, Lewy Bodies (LBs), the characteristic intra-neuronal inclusions found in sporadic PD brains, contain alpha-synuclein fibrils (Spillantini *et al.* 1998). These alpha-synuclein fibrils were initially thought to represent the cytotoxic species. However, evidence now suggests that the fibrils themselves are not the major

cytotoxic species, but that instead the soluble oligomers are the key damaging species (Paleologou *et al.*, 2009; Walsh *et al.*, 2002; El-Agnaf *et al.*, 2003; Kayed *et al.*, 2003; Sharon *et al.*, 2003). Recently, it has been shown that oligomers of alpha-synuclein, which are significantly more resistant to degradation with proteinase K (PK), and are therefore presumed to contain β-sheets, are capable of inducing higher aberrant levels of intercellular reactive oxygen species (ROS) than PK sensitive oligomers, indicating that the more PK resistant oligomers are the key damaging species (Cremades *et al.*, 2012).

Amyloid-beta (Abeta; Aβ) refers to 39 to 43 amino acid peptides which are derived by proteolytic cleavage from the amyloid precursor protein (APP). Amyloid-beta is a major component of proteinaceous plaques found in the brain tissue of Alzheimer's disease patients. Initially it was thought that amyloid-beta becomes cytotoxic when it forms large insoluble fabrillar aggregates but more recent studies have shown that small oligomers of amyloid-beta may be the major cytotoxic species. It has been hypothesized that these small oligomers bind to the neuronal membrane, leading to cell death, possibly by membrane permeabilization (Schauerte *et al.* 2010). By engineering a double cysteine mutant that stabilises the β-sheet structure through the formation of an intramolecular disulfide bond, it was shown that β-sheet-containing oligomers or protofibrillar species of Aβ42 were 50 times more potent inducers of neuronal apoptosis than amyloid fibrils or samples of monomeric wild-type Aβ42, in which toxic aggregates are only transiently formed (Sandberg *et al.* 2010).

Tau is a microtubule binding protein which can aggregate to form paired helical filaments (PHFs), which are amyloid in nature (based on cross β-sheet structure) and are the major constituent of neurofibrallar tangles in the neurons of Alzheimer diseased brains. Recent work shows that oligomeric tau aggregation intermediates are the most toxic compounds formed during the process of tau fibril formation. These oligomers effectively decrease cell viability and increase phospholipid vesicle leakage. (Flach *et al.* 2012) Tau oligomers have also been identified as an acutely toxic tau species in vivo, and induce neurodegeneration by affecting mitochondrial and synaptic function, both of which are early hallmarks in AD and other tauopathies (Lasagna-Reeves *et al.* 2011).

Amyloid fibrils are typically composed of 1000s of monomeric protein units. Such fibrils can be detected using the benzothiazole salt, thioflavin-T (ThT). Upon binding to β-sheet rich structures,, the fluorescence intensity of ThT increases by over three orders of magnitude, therefore making it an unusually sensitive and efficient reporter of amyloid structure (Maskevich *et al.*, 2007); its fluorescence quantum yield, $\Phi_{fluorescence}$, in water at room

temperature is 0.0001; however, when docked between surface side-chain grooves running parallel to the long axis of cross β-sheets, the fluorescence quantum yield increases dramatically. For ThT bound to amyloid fibrils of insulin $\Phi_{fluorescence}$ is 0.43 (LeVine, 1993; Sulatskaya *et al.*, 2010). However, assays to detect amyloid fibrils are usually performed in bulk on fluorimeters or plate-readers, which have low detection efficiencies and are only able to detect a signal after a significant proportion of monomer has formed fibrils. ThT in combination with total internal fluorescence (TIRF) microscopy has been used to study morphology changes of the large fibrilar species (Biancalana *et al.*, 2010; Andersen *et al.*, 2009; and Schauerte *et al.*, 2010), but these methods are again not suitable for determining levels of the smaller amyloidogenic oligomers, which are now understood to represent the major cytotoxic species in a number of neurodegenerative diseases as explained above.

The number of oligomers present in clinical samples represents only a small fraction of the total protein making their detection very challenging using ensemble techniques. Total levels of the specific proteins associated with neurodegenerative diseases have been studied using ELISA or bead-based cytometric assays, which have shown conflicting results. For example, total alpha-synuclein levels in cerebrospinal fluid (CSF) samples from PD patients compared with samples taken from healthy controls have been reported as being higher (Mollenhauer *et al.*, 2008; Tokuda *et al.*, 2006), or as being indiscernible (Ohrfelt *et al.*, 2009).

Methods have also been developed to detect the presence of oligomers in the CSF. These studies have been based on a novel dual ELISA method that is able to detect alpha-synuclein oligomers that are distinguishable from monomers, and show that aggregated alpha-synuclein is more abundant in the CSF from PD patients compared with healthy controls, Alzheimer's disease (AD) patients and progressive supranuclear palsy (PSP) patients (El-Agnaf *et al.* 2006; WO2011/033252). However, these methods rely on immunocapture to determine the mass of aggregated protein present, and are therefore limited by the inherent variability in antibody affinity for both capture and detection protocols. Effects such as the presence of protein mixtures, chaperone coating and post-translational modification may also prevent the proteins from being recognized by the detection antibodies (Schmid *et al.* 2013), due to the innate epitope based specificity of antibodies. Furthermore, only a fraction of the oligomers present will be detected (i.e. those containing alpha-synuclein), and other β-sheet oligomers not containing alpha-synuclein, will be unobservable. This is important, as there is evidence that the oligomeric species involved in the development of neurodegenerative diseases such as AD and PD may not be composed

of only one specific protein. Several studies suggest crossed-aggregations in which alpha-synuclein, tau or aβ form mixed-oligomers (Sierks *et al.*, 2011; Jensen *et al.*, 1997; Mandal *et al.*, 2006; Masliah *et al.*, 2001; Ono *et al.*, 2012). Moreover, oligomers are heterogeneous, varying in size and structure, and to date, it has not been possible to characterise the individual oligomer species detected in extracellular fluids. Due to these limitations of existing ELISA-based methods, an improved method for detecting oligomers involved in neurodegenerative disorders is needed.

Summary of the invention

The present invention provides a method for detecting the number and/or size of β-sheet containing amyloidogenic oligomers, for example in a cerebrospinal fluid (CSF), or peripheral blood plasma sample obtained from an individual. In particular, the method may be used to assess whether an individual has a neurodegenerative disorder, such as Parkinson's or Alzheimer's disease, for example before clinical symptoms associated with a neurodegenerative disorder appear.

Thus, in one aspect, the present invention provides a method of assessing whether an individual has a neurodegenerative disorder, the method comprising:

providing a cerebrospinal fluid (CSF) or peripheral blood plasma sample, preferably a CSF sample, obtained from the individual,

introducing the sample to a fluorescent dye,

measuring a change in one or more photophysical properties of the fluorescent dye upon binding of the fluorescent dye to individual β-sheet containing amyloidogenic oligomers in the sample, and

determining the number and/or size of β-sheet containing amyloidogenic oligomers in the sample from the measured change,

wherein the number and/or size of β-sheet containing amyloidogenic oligomers in the sample indicates whether the individual has a neurodegenerative disorder.

A higher number of β-sheet containing amyloidogenic oligomers in the sample, compared with the number of β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma sample obtained from a healthy individual, may indicates that the individual has a neurodegenerative disorder. Similarly, larger β-sheet containing amyloidogenic oligomers in the sample, compared with the size of β-sheet containing amyloidogenic oligomers in a CSF

or peripheral blood plasma sample obtained from a healthy individual, may indicates that the individual has a neurodegenerative disorder.

Preferably, the method comprises determining the number of β-sheet containing amyloidogenic oligomers in the cerebrospinal fluid (CSF) or peripheral blood plasma sample obtained from the individual.

Preferably, the β-sheet containing amyloidogenic oligomers appear as diffraction limited features when imaged using a microscope set up that has a diffraction limit of 300nm or less, more preferably the β-sheet containing amyloidogenic oligomers appear as diffraction limited features when imaged using a microscope set up that has a diffraction limit of 250nm or less.

The β-sheet containing amyloidogenic oligomers may have a diameter of 300nm or less, preferably 250nm or less, more preferably 50nm or less. In addition, the β-sheet containing amyloidogenic oligomers preferably have a diameter of at least 10nm. For example, the β-sheet containing amyloidogenic oligomers may have diameter of 10 to 50nm.

The β-sheet containing amyloidogenic oligomers may consist of up to 200 monomers, preferably up to 100 monomers. In addition, the β-sheet containing oligomers may consist of at least 4 monomers, preferably at least 10 monomers.

The β-sheet containing amyloidogenic oligomers comprise, or consist of, alpha-synuclein, amyloid-beta, and/or tau.

The photophysical property may be fluorescence intensity, fluorescence lifetime, Stokes shift, and/or polarization, preferably the photophysical property is fluorescence intensity and/or fluorescence lifetime, more preferably, the photophysical property is fluorescence intensity.

Measuring the change in the one or more photophysical properties may be performed using a sensitive microscope set up for single-molecule, or single particle microscopy. The single-molecule microscopy method is preferably single-molecule fluorescence microscopy. The microscope is preferably a confocal microscope, or a total internal fluorescence (TIRF) microscope.

The lens of the microscope may have a numerical aperture of 1.3 or higher. Preferably, the lens of the microscope has a numerical aperture of 1.3 to 1.49.

The microscope may comprises a light source, and the illumination intensity of the light source is 50 W/cm^2 or more. Preferably, the illumination intensity of the light source is 50 to 200 W/cm^2, more preferably 150 to 200 W/cm^2. The light source is preferably a laser, although an LED light source may also be used, for example. The microscope preferably comprises a single-photon detector, such as an electron multiplied charge coupled device (EMCCD); an avalanche photodiode detector (AVD); a complementary metal–oxide–semiconductor (CMOS); a scientific complementary metal–oxide–semiconductor (sCMOS); or a low level light detector.

The fluorescent dye preferably exhibits a Stokes shift of at least 20 nm, more preferably at least 50 nm. The fluorescent dye also preferably exhibits at least a 1,000 fold increase in fluorescence quantum yield upon binding to a β-sheet containing structure. The fluorescent dye is preferably, selected from the group consisting of: dimeric Thioflavin T, Thioflavin T, Thioflavin S, Thioflavin-glycol, Pittsburgh compound B (PiB), and analogues thereof. More preferably, the fluorescent dye is dimeric Thioflavin T or Thioflavin T. Most preferably, the fluorescent dye is Thioflavin T.

The microscope may be a TIRF microscope, and the sample may be diluted to achieve a separation of β-sheet containing amyloidogenic oligomers of at least 1 μm on the imaging surface of the microscope. Alternatively, the microscope may be a confocal microscope and the sample may be diluted such that there is a low probability that more than one β-sheet containing amyloidogenic oligomer is present in the probe volume at any one time. "Low probability" may refer to a probability of 1% or less, 0.1% or less, or 0.01% or less.

The individual may have been determined to be at risk of developing a neurodegenerative disorder. For example, the individual may have a family history of a neurodegenerative disorder. Preferably, the individual does not display any clinical symptoms associated with a neurodegenerative disorder. The neurodegenerative disorder is preferably characterized by protein aggregation.

In another aspect, the present invention provides a method of delaying or preventing the onset of clinical symptoms associated with a neurodegenerative disorder. The method may comprise:

(i) assessing whether or not an individual has a neurodegenerative disorder using a method as described herein; and

(ii) administering to an individual assessed as having a neurodegenerative disorder in (i) a therapeutically effective amount of an agent capable of delaying or preventing the onset of clinical symptoms associated with said neurodegenerative disorder.

Preferably, the method comprises administering to an individual assessed as having a neurodegenerative disorder in (i) a therapeutically effective amount of an agent which:

(a) directly or indirectly inhibits the formation of β-sheet containing amyloidogenic oligomers; or

(b) an agent that directly or indirectly induces the degradation of β-sheet containing amyloidogenic oligomers. As a further alternative, the method may comprise administering a therapeutically effective amount of an agent which binds to β-sheet containing amyloidogenic oligomers.

Also provided is an agent for use in a method of delaying or preventing the onset of clinical symptoms associated with a neurodegenerative disorder, the method comprising:

(i) assessing whether or not an individual has a neurodegenerative disorder using a method as described herein; and

(ii) administering to an individual assessed as having a neurodegenerative disorder in (i) a therapeutically effective amount of an agent that delays or prevents the onset of clinical symptoms associated with said neurodegenerative disorder.

In another aspect, the present invention provides a method of treating a neurodegenerative disorder, the method comprising assessing whether or not an individual has a neurodegenerative disorder using a method as described herein, and administering to an individual assessed as having a neurodegenerative disorder using said method a therapeutically effective amount of an agent that treats said neurodegenerative disorder.

The present invention also provides an agent for use in a method of treating a neurodegenerative disorder, the method comprising:

(i) assessing whether or not an individual has a neurodegenerative disorder using a method of the present invention; and

(ii) administering to an individual assessed as having a neurodegenerative disorder in (i) a therapeutically effective amount of an agent that treats said neurodegenerative disorder.

In another aspect, the present invention provides a method of identifying an agent capable of directly or indirectly inhibiting the formation of β-sheet containing amyloidogenic oligomers. The method may comprise:

(i) providing a solution comprising monomers capable of forming β-sheet containing amyloidogenic oligomers;

(ii) incubating said solution under conditions suitable for the formation of β-sheet containing amyloidogenic oligomers in the presence and absence of said agent;

(iii) introducing the solution to a fluorescent dye;

(iv) measuring a change in one or more photophysical properties of the fluorescent dye upon binding of the fluorescent dye to individual β-sheet containing amyloidogenic oligomers in the solution, and determining the number and/or size of β-sheet containing amyloidogenic oligomers in the solution from the measured change; and

(v) comparing the number and/or size of β-sheet containing amyloidogenic oligomers formed in the presence of the agent with the number and/or size of β-sheet containing amyloidogenic oligomers formed in the absence of said agent;

wherein a lower number of β-sheet containing amyloidogenic oligomers formed in the presence of the agent than in the absence of said agent indicates that the agent is capable of directly or indirectly inhibiting the formation of β-sheet containing amyloidogenic oligomers; and/or

wherein smaller β-sheet containing amyloidogenic oligomers formed in the presence of the agent than in the absence of said agent indicates that the agent is capable of directly or indirectly inhibiting the formation of β-sheet containing amyloidogenic oligomers.

Alternatively, the method may comprise:

(i) providing a CSF or peripheral blood plasma sample obtained from a non-human animal that is a model of a neurodegenerative disorder, wherein the animal has been treated with the agent;

(ii) introducing the sample to a fluorescent dye;

(iii) measuring a change in one or more photophysical properties of the fluorescent dye upon binding of the fluorescent dye to individual β-sheet containing amyloidogenic oligomers in the sample, and determining the number and/or size of β-sheet containing amyloidogenic oligomers in the sample from the measured change;

wherein the number and/or size of β-sheet containing amyloidogenic oligomers in the sample indicates whether the agent is capable of directly or indirectly inhibiting the formation of β-sheet containing amyloidogenic oligomers.

In a further aspect the present invention provides a method of identifying an agent capable of directly or indirectly inducing the degradation of β-sheet containing amyloidogenic oligomers. The method may comprise:

(i) providing a solution comprising β-sheet containing amyloidogenic oligomers;

(ii) incubating said solution in the presence and absence of said agent;

(iii) introducing the solution to a fluorescent dye;

(iv) measuring a change in one or more photophysical properties of the fluorescent dye upon binding of the fluorescent dye to individual β-sheet containing amyloidogenic oligomers in the solution, and determining the number and/or size of β-sheet containing amyloidogenic oligomers in the solution from the measured change;

; and

(iv) comparing the number and/or size of β-sheet containing amyloidogenic oligomers in the solution comprising the agent with the number and/or size of β-sheet containing amyloidogenic oligomers in the solution not containing the agent;

wherein a lower number of β-sheet containing amyloidogenic oligomers in the solution comprising the agent than in the solution not containing said agent indicates that the agent is capable of directly or indirectly inducing the degradation of β-sheet containing amyloidogenic oligomers;

and/or wherein smaller β-sheet containing amyloidogenic oligomers in the solution comprising the agent than in the solution not containing said agent indicates that the agent is capable of directly or indirectly inducing the degradation of β-sheet containing amyloidogenic oligomers.

In another aspect the present invention provides a method of identifying an agent capable of directly or indirectly inhibiting the degradation of β-sheet containing amyloid fibrils into β-sheet containing amyloidogenic oligomers. The method may comprise:

(i) providing a solution comprising β-sheet containing amyloid fibrils;

(ii) incubating said solution in the presence and absence of said agent;

(iii) introducing the solution to a fluorescent dye;

(iv) measuring a change in one or more photophysical properties of the fluorescent dye upon binding of the fluorescent dye to individual β-sheet containing amyloidogenic oligomers in the solution, and determining the number and/or size of β-sheet containing amyloidogenic oligomers in the solution from the measured change; and

(v) comparing the number of β-sheet containing amyloidogenic oligomers in the solution comprising the agent with the number of β-sheet containing amyloidogenic oligomers in the solution not containing the agent;

wherein a lower number of β-sheet containing amyloidogenic oligomers in the solution comprising the agent than in the solution not containing said agent indicates that the agent is capable of directly or indirectly inhibiting the degradation of β-sheet containing amyloid fibrils into β-sheet containing amyloidogenic oligomers

In another aspect the present invention provides a method of identifying an agent capable of reducing the number and/or size of β-sheet containing amyloidogenic oligomers in an individual. The method may comprise:

(i) determining the number and/or size of β-sheet containing amyloidogenic oligomers in a cerebrospinal fluid (CSF) or a peripheral blood plasma sample obtained from the individual using a method as described herein;

(ii) administering the agent to the individual;

(iii) determining the number and/or size of β-sheet containing amyloidogenic oligomers in a cerebrospinal fluid (CSF) or a peripheral blood plasma sample obtained from the individual using using a method as described herein; and

(iv) comparing the number and/or size of β-sheet containing amyloidogenic oligomers determined in (i) with the number and/or size of β-sheet containing amyloidogenic oligomers determined in (iii);

wherein a lower number of β-sheet containing amyloidogenic oligomers following administration of the agent indicates that the agent is capable of directly or indirectly reducing the number of β-sheet containing amyloidogenic oligomers in an individual; and/or

wherein smaller β-sheet containing amyloidogenic oligomers following administration of the agent indicates that the agent is capable of directly or indirectly reducing the size of β-sheet containing amyloidogenic oligomers in an individual.

In a further aspect, the present invention relates to an *in vitro* method for determining the number and/or size of β-sheet containing amyloidogenic oligomers in a sample, such as a cerebrospinal fluid (CSF) or a peripheral blood plasma sample. The method may comprise:

providing a sample,

introducing the sample to a fluorescent dye,

measuring a change in one or more photophysical properties of the fluorescent dye upon binding of the fluorescent dye to individual β-sheet containing amyloidogenic oligomers in the sample, and

determining the number and/or size of β-sheet containing amyloidogenic oligomers in the sample from the measured change.

Other aspects of the invention, and various embodiment of the invention, are as described herein.

Brief Description of the Figures

Figure 1 A: Structure of thioflavin-T (ThT). In the unbound state, ThT has a low fluorescence intensity; however, when bound to β-sheet containing oligomers or fibrils, the fluorescence quantum yield increases significantly (50,000 fold). **B:** ThT fluorescence images using TIRF microscopy of 700 pM (starting monomer concentration) samples taken after 0, 2 and 5 days of fibril formation. Toxic β-sheet structures are selectively visualized. Arrows indicate example oligomers. **C:** Corresponding AF647 fluorescence images for the same sample. Monomers and oligomers were detected in this channel, with brighter aggregates correlating well with ThT fluorescence. Arrows show the same oligomers detected in the AF647 channel. Scale bars in fluorescence images are 10 μm in length. **D:** Time-progression of ThT active species in the same sample over three days detected using single-molecule confocal microscopy. **E:** Time-progression of ThT active species over 30 hours detected using TIRF microscopy. Mean oligomer counts from three aggregations are shown. Error bars represent standard deviation.

Figure 2 A: ThT fluorescence image from 100 nM (monomer equivalent) sample of enriched oligomers. As expected, large numbers of β-sheet structures are observed. Scale bar in fluorescence image is 10 μm in length. **B:** atomic force microscopy (AFM) of enriched oligomers. **C:** dynamic light scattering (DLS) of monomer, enriched oligomers, and fibrils. The size of each of these species is indicated. **D:** circular dichroism (CD) spectra of monomer, enriched oligomers and fibrils.

Figure 3 A: Photon count intensity distribution histograms from two representative samples (Parkinson's disease (PD) (grey) – Case 10, healthy control (white) – Case 23) using dye ThT. These cases gave rise to counts similar to average for the PD and healthy control samples, respectively. The photon count is thought to be proportional to the size of the oligomer. **B:** Representative total internal refection fluorescence microscopy (TIRF) images from Case 10 and Case 23. For each sample, 27 images were taken (three 3 × 3 grid scans). For Case 10, a total of 1201 oligomers were detected, whereas for the healthy control, a total of 348 oligomers were detected from the 27 images. Squares show detected oligomers, and the scale bar is 8.7 μm in length. **C:** A histogram of oligomer counts for each sample using dye ThT, grey bars are from PD patients, and white from healthy controls

(HC). **D:** Box plots of the same data for the number of oligomers detected. Horizontal lines show the mean counts for PD and HC samples. **E:** A histogram of oligomer counts for each sample using dye diThT-PEG, dark grey bars are from PD patients, and pale grey bars from healthy controls (HC).

Figure 4A: Structure of ThT. B. Structure of di-ThT-PEG2. C. Detected spots from ThT bound to alpha-synuclein oligomers (synthetic) (5 μM ThT). D. Detected spots from di-ThT-PEG2 bound to alpha-synuclein oligomers (synthetic) (5 μM di-ThT-PEG2). E. Intensity distribution histograms from ThT and di-ThT-PEG2 bound oligomers from a solution of synthetic oligomers. F. Intensity distribution histograms from ThT and di-ThT-PEG2 bound oligomers in a CSF sample. Intensities are higher with di-ThT-PEG2.

Figure 5: Fluorescence lifetime image showing alpha-synuclein oligomers bound to ThT. A 350 nM solution of aggregated alpha-synuclein was diluted into 20 μM ThT. The scale bar is 10 μm in length.

Figure 6 A: Structure of dyes thioflavin-T (ThT), di-ThT-PEG2 and DCVJ. **B**: Density alpha-synuclein oligomers detected with ThT, diTHT-PEG and DCVJ at given alpha-synuclein concentrations. Each point represents the mean of three repeats. The standard deviation is represented by error bars. Each repeat consisted of the average density of 27 field of views. di-ThT had a higher detection sensitivity than ThT. Use of DCVJ resulted in the "detection" of very high densities of oligomers at high dye concentrations. This was found to be the result of a very high false positive rate for this dye. Use of dyes BTA-1 and CR was also tested but these dyes were found not to be useful in this particular setting, as they showed variable or no detection of alpha-synuclein oligomers. The concentration of alpha-synuclein was optimized to provide an optimal density and was kept at 70 nM throughout the experiment. All data were normalized to the asymptote of the sigmoidal to guide the eye.

Figure 7: Detection of fluorescent puncta in peripheral blood plasma from a PD patient. Despite the higher autofluorescence background in peripheral blood plasma as compared with other biofluids, such as CSF, fluorescent puncta of varying brightness were readily detected (highlighted with white squares in Figure 7). The scale bar is 5μM. The detection of fluorescent puncta is consistent with the detection of β-sheet containing amyloidogenic oligomers.

Detailed description of the invention

The present invention provides a method for detecting the number and/or size of β-sheet containing amyloidogenic oligomers, which may be used to assess whether an individual has a neurodegenerative disorder, such as Parkinson's or Alzheimer's disease, in particular before clinical symptoms associated with neurodegenerative disorders appear. Specifically, the present inventors have found that use of a fluorescent dye which exhibits a change in one or more photophysical properties upon binding to a β-sheet containing amyloidogenic oligomer, as described in further detail below, allows the number and/or size of β-sheet containing amyloidogenic oligomers to be determined. Oligomers of alpha-synuclein, presumed to contain β-sheets, for example, have previously been identified as the major cytotoxic species in the development of Parkinson's disease as mentioned above (Cremades *et al.*, 2012). That these oligomers do indeed contain β-sheets has now been confirmed by the present inventors.

The method of the invention has major advantages over previously described ELISA-based assays for the detection of amyloidogenic oligomers, as ELISA-based methods are specific to a particular amyloidogenic protein, whereas the fluorescent dyes employed in the methods of the present invention allow any amyloidogenic oligomer comprising a common β-sheet structure to be detected. Another disadvantage of ELISA-based methods is that they may not detect all amyloidogenic oligomers present in a sample as a result of post-translational modification, chaperone or lipid coating, or buried epitopes, which can prevent binding of antibodies to the oligomers. Furthermore, the ELISA-based methods for the detection of alpha-synuclein oligomers disclosed in WO2012/033252 and El-Agnaf *et al.* (2006), which are described as able to detect the amount of soluble alpha-synuclein oligomers in a sample, cannot distinguish between oligomers comprising β-sheets and those that do not. Specifically, these methods involve a sandwich ELISA in which the same antibody is used in both the capture and detection steps, thereby only detecting alpha-synuclein oligomers, and not monomers, as only alpha-synuclein oligomers comprise two epitopes in the same structure. However, the inability to specifically detect β-sheet containing oligomers makes these methods unsuitable for determining the amount or number of the key cytotoxic oligomer species involved in Parkinson's disease, for example. In addition, ELISA-based methods rely on the use of antibodies, which can result in problems as antibodies may bind non-specifically resulting in false positive results, or may not bind at all if the epitope is blocked, for example by molecular chaperones, mixed aggregates, or post-translational modifications.

The term "amyloidogenic oligomer" preferably refers to an oligomer capable of forming amyloid fibrils and comprising a β-sheet motif which can be bound by Thioflavin T, for example. Amyloid fibrils are insoluble fibrous protein aggregates comprising a β-sheet motif.

The fluorescent dyes employed in the methods of the present invention, such as ThT, allow individual β-sheet oligomers of any protein composition to be detected, as well as allowing the number and/or size of such oligomers present in e.g. a CSF or peripheral blood plasma sample to be determined. In contrast, ELISA-based techniques simply estimate the total amount of a specific protein, such as alpha-synuclein, that is present in oligomer form in a sample (i.e. a similar signal would be observed if one large oligomer was present in a sample, versus two smaller ones), which means that the number of oligomers of the protein in question present in the sample cannot be determined.

As mentioned above, samples that can be analysed using the methods of the present invention include CSF and peripheral blood plasma samples. Preferably, the sample is a CSF sample. CSF samples from Parkinson's disease patients, for example, have been shown by the present inventors to contain β-sheet containing amyloidogenic oligomers. These oligomers appear as diffraction limited spots if imaged using, for example, a total internal reflection fluorescence (TIRF) single-molecule microscope with a diffraction limit of 300nm or less. CSF samples only contain very few, if any, amyloidogenic fibrils, and these in any case appear larger than diffraction limited spots when imaged using such a TIRF microscope. In addition, the number and/or size of β-sheet containing amyloidogenic oligomers present in a sample obtained from an individual has been shown by the present inventors to be indicative of whether the individual has a neurodegenerative disorder.

Thus, a method of assessing whether an individual has a neurodegenerative disorder according to the invention may comprise determining the number and/or size of β-sheet containing amyloidogenic oligomers in a CSF or a peripheral blood plasma sample obtained from the individual, wherein the number and/or size of β-sheet containing amyloidogenic oligomers in the sample indicates whether the individual has a neurodegenerative disorder.

The present inventors have shown that the number and/or size of β-sheet containing amyloidogenic oligomers in CSF samples obtained from individuals with a neurodegenerative disorder, such as Parkinson's disease, is elevated compared with the number and/or size of β-sheet containing amyloidogenic oligomers in CSF samples obtained

from age-matched healthy individuals. Thus, a higher number of β-sheet containing amyloidogenic oligomers in a CSF sample or peripheral blood plasma sample obtained from an individual, compared with the number of β-sheet containing amyloidogenic oligomers in a CSF sample or peripheral blood plasma sample obtained from a healthy individual, may indicate that the individual has a neurodegenerative disorder. In addition, or alternatively, larger β-sheet containing amyloidogenic oligomers in a CSF sample or peripheral blood plasma sample obtained from an individual, compared with the size of β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma sample obtained from a healthy individual, may indicate that the individual has a neurodegenerative disorder.

The number of β-sheet containing amyloidogenic oligomers may refer to the concentration of β-sheet containing amyloidogenic oligomers. The size of the β-sheet containing amyloidogenic oligomers may refer to the relative size of the β-sheet containing amyloidogenic oligomers, e.g. compared with the size of the β-sheet containing amyloidogenic oligomers in a sample obtained from a healthy individual. The size of β-sheet containing amyloidogenic oligomers preferably refers to the size of individual β-sheet containing amyloidogenic oligomers.

Preferably, a method of the present invention comprises determining the number of β-sheet containing amyloidogenic oligomers, e.g. in a cerebrospinal fluid (CSF) or peripheral blood plasma sample.

Alternatively, the same, or a lower, number of β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma sample obtained from an individual, compared with the number of β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma sample obtained from a healthy individual, may indicate that the individual does not have a neurodegenerative disorder. Similarly, smaller β-sheet containing amyloidogenic oligomers, or β-sheet containing amyloidogenic oligomers of the same size, in a CSF sample or peripheral blood plasma sample obtained from an individual, compared with the size of β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma sample obtained from a healthy individual, may indicate that the individual does not have a neurodegenerative disorder.

For example, a number of β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma sample obtained from an individual which is at least 1.5 fold, at least 2-fold, at least 2.5-fold, at least 3-fold , at least 3.5-fold, at least 4-fold, at least 4.5-fold,

at least 5-fold, at least 5.5-fold, at least 6-fold, at least 6.5-fold, at least 7-fold, at least 7.5-fold, at least 8-fold, at least 8.5-fold, at least 9-fold, at least 9.5-fold, or at least 10-fold, preferably at least 3-fold, higher than the number of β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma sample obtained from a healthy individual may indicate that the individual has a neurodegenerative disorder.

In addition, or alternatively, β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma sample obtained from an individual which are at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 110%, at least 120%, at least 130%, at least 140%, or at least 150% larger than the β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma sample obtained from a healthy individual may indicate that the individual has a neurodegenerative disorder. The size of β-sheet containing amyloidogenic oligomers may refer to the average size, or median size, of the β-sheet containing amyloidogenic oligomers. Thus, for example, the presence of β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma sample obtained from an individual with an average size, or median size, which is at least 50% larger than the average size of β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma sample obtained from a healthy individual, may indicate that the individual has a neurodegenerative disorder. In addition, or alternatively, the size of β-sheet containing amyloidogenic oligomers may refer to the diameter of the β-sheet containing amyloidogenic oligomers.

A healthy individual may be an individual who does not have a neurodegenerative disorder, such as Parkinson's disease or Alzheimer's disease. The healthy individual may be age-matched to the individual being assessed for the presence of a neurodegenerative disorder. A healthy individual may also be referred to as a normal individual, or a healthy control.

The β-sheet containing amyloidogenic oligomers may have a diameter of 300nm or less, 250nm or less, 200nm or less, 150nm or less, 100nm or less, or 50nm or less, 40nm or less, 30nm or less, 20nm or less, or 10nm or less. Preferably, the β-sheet containing amyloidogenic oligomers have a diameter of 250nm or less, more preferably 50nm or less. The β-sheet containing amyloidogenic oligomers may also have a diameter of at least 10nm. Preferably, the β-sheet containing amyloidogenic oligomers have a diameter of 10 to 250nm, more preferably 10 to 50nm. The β-sheet containing amyloidogenic oligomers have a

diameter of about 10nm. The diameter of the β-sheet containing amyloidogenic oligomers may be determined by DLS, or electron microscopy, for example.

In addition, or alternatively, the β-sheet containing amyloidogenic oligomers may appear as diffraction limited features when imaged using a microscope set up that has a diffraction limit of 300nm or less, 250nm or less, 200nm or less, or 180nm or less. Preferably, a diffraction limit of the microscope set up is 250nm or less.

In addition, or alternatively, the β-sheet containing amyloidogenic oligomers may be 4mers to 200mers, in other words the β-sheet containing amyloidogenic oligomers may consist of 4 to 200 monomers each. A 4mer is needed in order for dyes such as ThT to be able to bind to the amyloidogenic oligomer. The β-sheet containing amyloidogenic oligomers may consist of 200 monomers or less, 190 monomers or less, 180 monomers or less, 170 monomers or less, 160 monomers or less, 150 monomers or less, 140 monomers or less, 130 monomers or less, 120 monomers or less, 110 monomers or less, 100 monomers or less, 90 monomers or less, 80 monomers or less, 70 monomers or less, 60 monomers or less, or 50 monomers or less. Preferably, the β-sheet containing amyloidogenic oligomers consists of 150 monomers or less. More preferably, the β-sheet containing amyloidogenic oligomers consists of 100 monomers or less. In addition, the β-sheet containing amyloidogenic oligomers may consist of at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100 monomers. In a particular example, the β-sheet containing amyloidogenic oligomers may be 4mers to 200mers, 4mers to 190mers, 4mers to 180mers, 4mers to 170mers, 4mers to 160mers, 4mers to 150mers, 4mers to 140mers, 4mers to 130mers, 4mers to 120mers, 4mers to 110mers, 4mers to 100mers, 4mers to 90mers, 4mers to 80mers, 4mers to 70mers, 4mers to 60mers, or 4mers to 50mers, i.e. consist of 4 to 200, 4 to 190, 4 to 180, 4 to 170, 4 to 160, 4 to 150, 4 to 140, 4 to 130, 4 to 120, 4 to 110, 4 to 100, 4 to 90, 4 to 80, 4 to 70, 4 to 60, or 4 to 50 monomers.

Alternatively, the β-sheet containing amyloidogenic oligomers may be 10mers to 200mers, 10mers to 190mers, 10mers to 180mers, 10mers to 170mers, 10mers to 160mers, 10mers to 150mers, 10mers to 140mers, 10mers to 130mers, 10mers to 120mers, 10mers to 110mers, 10mers to 100mers, 10mers to 90mers, 10mers to 80mers, 10mers to 70mers, 10mers to 60mers, or 10mers to 50mers, i.e. consist of 10 to 200, 10 to 190, 10 to 180, 10 to 170, 10 to 160, 10 to 150, 10 to 140, 10 to 130, 10 to 120, 10 to 110, 10 to 100, 10 to 90, 10 to 80, 10 to 70, 10 to 60, or 10 to 50 monomers. Preferably, the β-sheet containing

amyloidogenic oligomers are 4mers to 150mers. More preferably, the β-sheet containing amyloidogenic oligomers are 10mers to 150mers. More preferably still, the β-sheet containing amyloidogenic oligomers are 4mers to 100mers. Most preferably, the β-sheet containing amyloidogenic oligomers are or 10mers to 100mers.

The β-sheet containing amyloidogenic oligomers are preferably soluble. Soluble in this context refers to the oligomers which are soluble in water. Amyloid fibrils, in contrast, are insoluble in water.

The β-sheet containing amyloidogenic oligomers may comprise or consist of any β-sheet containing oligomers amyloidogenic protein associated with a neurodegenerative disorder.

Preferably, β-sheet containing amyloidogenic oligomers are β-sheet containing oligomers of alpha-synuclein, amyloid-beta, and/or tau. The β-sheet containing amyloidogenic oligomers may comprise, or consist of, alpha-synuclein, amyloid-beta, and/or tau. For example, the β-sheet containing amyloidogenic oligomers may comprise, or consist of, alpha-synuclein and/or amyloid-beta. As a further example, the β-sheet containing amyloidogenic oligomers may comprise, or consist of, alpha-synuclein. Alternatively, the β-sheet containing amyloidogenic oligomers may comprise, or consist of, amyloid-beta and/or tau. The β-sheet containing amyloidogenic oligomers may comprise, or consist of, amyloid-beta. As a further alternative, the β-sheet containing amyloidogenic oligomers may comprise, or consist of tau. Specifically, where the neurodegenerative disorder is Parkinson's disease, the β-sheet containing amyloidogenic oligomers preferably comprise, or consist of, alpha-synuclein and/or amyloid-beta, most preferably alpha-synuclein. Where the neurodegenerative disorder is Alzheimer's disease, the β-sheet containing amyloidogenic oligomers may comprise, or consist of, amyloid-beta, alpha-synuclein, and/or tau, preferably the β-sheet containing amyloidogenic oligomers comprise, or consist of amyloid-beta and optionally tau. A β-sheet containing amyloidogenic oligomer comprising or consisting of a particular protein, such as alpha-synuclein, may also be referred to as a β-sheet containing amyloidogenic oligomer of alpha-synuclein.

Alternatively, the β-sheet containing amyloidogenic oligomers may comprise or consist of prion protein (PrP^{Sc}), superoxide dismutase (SOD1), TAR DNA binding protein (e.g. TDP-43, or TARDBP), or RNA-binding protein FUS (FUS).

The methods of the present invention comprise determining the number and/or size of β-sheet containing amyloidogenic oligomers using a fluorescent dye which exhibits a change

in one or more photophysical properties upon binding to a β-sheet containing amyloidogenic oligomer . The photophysical property may be fluorescence intensity, fluorescence lifetime, Stokes shift, and/or polarization. Preferably, the photophysical property is fluorescence intensity and/or fluorescence lifetime, most preferably fluorescence intensity.

As mentioned above, the polarisation of the fluorescent dye may change upon binding to a β-sheet containing amyloidogenic oligomer. A change in the polarization of the fluorescent dye may refer to a change in the polarization of light emitted by the fluorescent dye. Changes in the polarization of the dye may be measured by measuring fluorescence anisotropy, also referred to as polarization anisotropy. Methods for measuring fluorescence anisotropy are well-known in the art and include single-molecule microscopy, such as single-molecule fluorescence microscopy, as described elsewhere herein. Suitable methods are also described in Gradinaru *et al.* (2010), for example. Detecting a change in the polarization of the dye is particularly useful in the context of determining the number of β-sheet containing amyloidogenic oligomers.

In addition, or alternatively, the Stokes shift of the fluorescent dye may change upon binding to a β-sheet containing amyloidogenic oligomer. Thus, an unbound dye molecule may exhibit a different Stokes shift compared with the Stokes shift of a dye molecule bound to a β-sheet containing amyloidogenic oligomer. A change in the Stokes shift may be determined using fluorescence microscopy, e.g. single molecule fluorescence microscopy, as described elsewhere herein. Detecting a change in the Stokes shift of a dye is particularly useful in the context of determining the number of β-sheet containing amyloidogenic oligomers.

In addition, or alternatively, the fluorescence lifetime of the fluorescent dye may change upon binding to a β-sheet containing amyloidogenic oligomer. Preferably, the fluorescence lifetime of the fluorescent dye increases upon binding to a β-sheet containing amyloidogenic oligomer. Fluorescence lifetime may be measured using fluorescence-lifetime imaging microscopy (FLIM). Methods for determining fluorescence lifetime are well-known in the art and include single-molecule microscopy, such as single-molecule fluorescence microscopy as described elsewhere herein. Detecting a change in the fluorescence lifetime of the fluorescent dye is particularly useful in the context of determining the number of β-sheet containing amyloidogenic oligomers. The change in fluorescence lifetime is preferably an increase in fluorescence lifetime. For example, the fluorescence lifetime of the dye may increase at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, or at least 10-fold upon binding to a β-sheet containing

amyloidogenic oligomer. For example, the fluorescence lifetime of the dye may increase from 0.5 ns to 1-2 ns upon binding to a β-sheet containing amyloidogenic oligomer.

In addition, or alternatively, the fluorescence intensity of the fluorescent dye may change upon binding to a β-sheet containing amyloidogenic oligomer. Preferably, the fluorescent dye exhibits and increase in fluorescence intensity upon binding to a β-sheet containing amyloidogenic oligomer. Methods for determining fluorescence intensity are also well-known in the art and include single-molecule microscopy, such as single-molecule fluorescence microscopy as described herein

As mentioned above, a change in the photophysical property of the fluorescent dye, such as fluorescence intensity, fluorescence lifetime, Stokes shift, and/or polarization, may be measured using single-molecule microscopy, such as single-molecule fluorescence microscopy. Thus, in addition to a fluorescent dye which exhibits a change in one or more photophysical properties upon binding to a β-sheet containing amyloidogenic oligomer, a method of the invention may comprise the use of a microscope capable of single-molecule microscopy. The single-molecule microscopy is preferably single-molecule fluorescence microscopy, more preferably total internal reflection fluorescence microscopy (TIRFM), or single-molecule confocal microscopy. Most preferably, the single-molecule microscopy is TIRFM. Confocal microscopy and TIRFM are well-known in the art. Suitable confocal microscopy techniques are Horrocks *et al.* (2011), and Moerner *et al.* (2003), while suitable TIRFM techniques are described in Narayan *et al.* (2013), Fish (2009), and Moerner *et al.* (2003).

The microscope preferably has a lens with a numerical aperture of 1.30 or higher, 1.35 or higher, 1.40 or higher, 1.41 or higher, 1.42 or higher, 1.43 or higher, 1.44 or higher, 1.45 or higher, 1.46 or higher, 1.47 or higher, 1.48 or higher, or 1.49 or higher. The numerical aperture may be 1.49 or less. Specifically, the numerical aperture may be 1.30 to 1.49, 1.35 to 1.49, 1.40 to 1.49, 1.41 to 1.49, 1.42 to 1.49, 1.43 to 1.49, 1.44 to 1.49, 1.45 to 1.49, 1.46 to 1.49, 1.47 to 1.49, or 1.48 to 1.49.

The microscope preferably comprises a light source with an illumination intensity of 20 W/cm^2 or more, 50 W/cm^2 or more, 100 W/cm^2 or more, or 150 W/cm^2 or more. The illumination intensity of the light source is preferably 200 W/cm^2 or less. The illumination intensity of the light source may be 20 to 200 W/cm^2, preferably, 50 to 200 W/cm^2. Most preferably, the illumination intensity of the light source is 150 to 200 W/cm^2.

The light source may be an LED light source or a laser. Preferably the light source is a laser. The laser is preferably a continuous wave laser.

The excitation wavelength of the light source is chosen based on the fluorescent dye employed in the method of the invention. The excitation wavelength is preferably in the range of 300 to 900nm. Where the dye is ThT or a ThT analogue, such as diThT-PEG2, the excitation wavelength of the light source is preferably in the range of 350-500nm. The excitation wavelength for ThT may be 405nm.

The microscope preferably comprises a single-photon detector, such as an electron multiplied charge coupled device (EMCCD), an avalanche photodiode detector (AVD), a complementary metal–oxide–semiconductor (CMOS), a scientific complementary metal–oxide–semiconductor (sCMOS), or a low level light detector. The low level light detector may be a photomultiplier (PMT) detection system.

A sample, such as a cerebrospinal fluid (CSF) or peripheral blood plasma sample, may be diluted to facilitate imaging of individual oligomers. For example, the sample may be diluted to achieve a separation of β-sheet containing amyloidogenic oligomers of at least 1 μm, at least 2 μm, at least 3 μm, at least 4 μm, or at least 5 μm on an imaging surface of the microscope. Preferably, the sample is diluted to achieve a separation of β-sheet containing amyloidogenic oligomers of at least 1 μm on an imaging surface of the microscope. Alternatively, the sample may be diluted such that there is a low probability that more than one β-sheet containing amyloidogenic oligomer is present in the probe volume at any one time. A low probability may refer to a probability of 1% or less, 0.1% or less, 0.01% or less, or 0.001% or less. Specifically, where the microscope is a TIRF microscope, the sample is preferably diluted to achieve a separation of β-sheet containing amyloidogenic oligomers of at least 1 μm on the imaging surface of the TIRF microscope. Where the microscope is a confocal microscope, the sample is preferably diluted such that there is a low probability that more than one β-sheet containing amyloidogenic oligomer is present in the probe volume at any one time.

The fluorescent dye may exhibit a Stokes shift of at least 20 nm, at least 30 nm, at least 40 nm, at least 50 nm, at least 60 nm, at least 70nm, at least 80nm, at least 90nm, or at least 100nm. Preferably, the fluorescent dye exhibits a Stokes shift of at least 50 nm. A large Stokes shift is useful as it allows fluorescence from background impurities, which tend to

have a lower Stokes shift, to be effectively filtered out. A method of the invention may therefore include Stokes shift filtering of the fluorescent signal emitted by the sample. Stokes shift filtering may be achieved through the use of appropriate filters, for example.
The dye used in the methods of the present invention is preferably a fluorescent dye. As mentioned above, the fluorescent dye may exhibit a change, such as an increase, in fluorescence intensity upon binding to β-sheet containing structures or an increase in fluorescence lifetime. The dye may have a binding affinity for β-sheet containing structures, such as β-sheet containing amyloidogenic oligomers, over other structural forms.

Dyes having these characteristics are well known in the art, and the fluorescent dye thioflavin T (ThT) is frequently used as a dye for binding to amyloid fibrils, including amyloid beta peptide aggregates (see Qin *et al.,* 2010). Dyes such as thioflavin also bind to alpha-synuclein aggregates, including β-sheet containing alpha-synuclein and tau oligomers and fibrils.

The binding of the fluorescent dye to a β-sheet containing structure is associated with a change in fluorescence quantum yield. Thus, the fluorescence intensity of the dye may increase upon binding, as may be determined from quantum yield measurements for the free dye compared to the bound dye. The fluorescent dye may exhibit at least a 100-fold, 1,000-fold or 10,000-fold increase in fluorescent quantum yield upon binding to a β-sheet containing structure, such as an oligomer.

Without wishing to be bound by theory, the present inventors believe that molecular rotor-type dyes exhibit such increases, as the binding event limits or prevents rotation of rings within the dye (such as the relative rotation of aniline and benzathiole rings in thioflavin dyes). In the unbound state, the rotation of rings with the dye gives rise to quenching in the excited state, which results in very poor fluorescence. A dye, such as ThT, is understood to bind within surface side-chain grooves running parallel to the long axis of cross β-sheets. In doing so rotation is prevented, and the fluorescence quantum yield increases dramatically.

For example, ThT has a fluorescent quantum yield, $\Phi_{fluorescence}$, of 0.0001 as a free dye in water at room temperature (20°C) and a yield of 0.43 when bound to the β-sheet containing amyloid fibrils of insulin.

The dye may have affinity for β-sheet containing structures over other structures, for example β-sheet containing oligomers over monomeric forms. The dye may bind to a β-sheet containing structure, such as a β-sheet containing oligomer, with a dissociation constant of at most 10 μM, at most 5 μM, at most 1 μM, at most 0.5 μM, at most 0.1 μM, or at most 0.05 μM.

The dye may possess low affinity for monomeric species such as monomeric alpha-synuclein, and monomeric amyloid-beta, and/or monomeric tau. The dissociation constant for a dye binding to a monomeric species may be at least 10-fold, at least 100-fold, or at least 1,000-fold greater than the dissociation constant for the dye bound to oligomeric and fibril β-sheet containing aggregations of the monomeric species.

The determination of a dissociation constant for a dye binding to monomeric and aggregate species is common in the art. Example determinations are described in Qin *et al.* (2010) for dyes binding to amyloid-beta aggregates.

For example, ThT binds to β-sheet containing aggregates of Aβ40 with a dissociation constant of around 1 μM. ThT exhibits no detectable affinity for the monomeric form of Aβ40.

The dye may have an affinity for β-sheet containing structures over other structural types. The β-sheet binding specificity may be expressed as a signal-to-noise ratio at the fluorescent emission wavelength for a specified concentration of β-sheet containing material. Qin *et al.* (2010) report that ThT has a signal-to-noise ratio of 2 at a concentration of 15 μM of amyloid-beta aggregate at 485 nm.

A dye for use in the present invention will typically have a signal-to-noise ratio of 2 at a concentration of β-sheet containing structure of at most 50 μM, at most 25 μM, at most 15 μM, at most 10 μM or at most 5 μM.

Dyes such as ThT and its analogues may be used in the present invention. Thus, the dye may be a fluorescent dye comprising one or more benzothiazole groups. A benzothiazole group may be substituted at the 2-position with aniline, including substituted anilines, such as those where the amine group is an alkyl amine, such as methylamine and dimethylamine. Such dyes may be broadly referred to as thioflavin dyes.

The benzothiazole is preferably a quaternary, or charged, benzothiazole group. Thus, the nitrogen ring atom at the 3-position is substituted, for example is substituted with alkyl, such as methyl. Where the benzothiazole is charged, a suitable counter anion is provided, such as halide, such as chloride. Charged benzothiazoles are preferred over neutral benzothiazoles as the latter may not exhibit an increase in fluorescence intensity upon binding to β-sheet containing aggregates.

The benzothiazole may be optionally substituted on the benzene ring. Typically such dye compounds are substituted at the 6-position. Example optional substituents include alkyl, such as methyl, and alkoxy, such as methoxy and pentoxy, and substituted alkoxy, including alkoxy alkoxy, such as ethoxy ethoxy. Additionally, the benzene ring may be optionally substituted with sulfonic acid, or its sulfonate salt from. Where a sulfonic acid group is present it may be a substituent at the 7-position.

The dye ThT may be regarded as a benzothiazole containing compound where the 2-position is substituted with dimethylaniline, the 3-position is substituted with methyl (and the counter anion may be chloride) and the 6-position is substituted with methyl.

Thioflavin dyes having a range of benzene substituent groups are described by Qin *et al.*, and the exemplified dyes PEG2-ThT, MeO-ThT and C5-ThT all find used in the methods of the present case. The dye thioflavin S also finds use in the present case. This dye is a ThT compound having a sulfonate group as a substituent to the 7-position of the benzothiazole.

Qin *et al.* (2010) have previously suggested that neutral thioflavin dyes do not show a fluorescent change on amyloid binding. The present inventors believe that there is a change in fluorescent binding with neutral thioflavin dyes, and that this change is small and is in principle detectable.

A dye may have two benzothiazole groups, such as two thioflavin groups, which are covalently linked, either directly or *via* a linker group. Such dyes may be broadly referred to as dimer dyes. A linker group may be an alkylene linker or a hetereoalkylene linker, such as an alkylene glycol linker, including a polyalkylene glycol linker. A benzothiazole group may be linked to another benzothiazole group *via* the 6-position or the 1-position.

Qin *et al.* (2010) also describe dye compounds having two thioflavin groups connected via a heteroalkylene linker. Here, one ThT group is linked *via* the 6-position to the 6-position of another ThT group by a polyethylene glycol linker. In another example, one ThT group is linked *via* the 6-position to the aniline nitrogen of another ThT by a polyethylene glycol linker.

The dimer dyes described by Qin *et al.* (2010), such as diThT-PEG2 and diThT-PEG2-HT, find use in the methods of the present case.

Dimer dyes are reported to have improved binding affinities for β-sheet containing structures compared to the monomer forms, whilst retaining specificity for β-sheet containing oligomers and fibrils over structures lacking this structural motif. The dimers also exhibit an increase in fluorescence intensity on binding to a β-sheet structure.

The dye for use in the methods described herein is not limited to a dye having a benzothiazole group. Other dyes are known to have affinity for β-sheet containing structures, and to exhibit an increase in fluorescence intensity upon binding. An alternative dye to ThT, for example, is 9-(2,2-Dicyanovinyl)julolidine (DCVJ).

As will be apparent from the discussion above, the dye is a detectable label that binds non-covalently to β-sheet containing structures.

In the methods described herein, the dye is used at a concentration that is appropriate for maximum detection efficiency. At high dye concentrations the detection efficiency decreases, since the integrated fluorescence background increases linearly to a value higher in magnitude than the limited fluorescence signal generated from a dye-bound oligomer,, in addition non-specific binding can be observed at higher concentrations of dye, increasing the false-positive rate. Consequently, at higher dye concentrations the signal-to-noise ratio decreases. In one embodiment, the dye is used at a concentration of at most 1 μM, at most 5 μM or at most 10 μM.

The dye is preferably selected from the group consisting of: dimeric Thioflavin T, such as diThT-PEG2, Thioflavin T, Thioflavin S, Thioflavin-glycol, such as PEG2-ThT, and Pittsburgh compound B (PiB), 2-(4'-(Dimethylamino)phenyl)-6-methyl-benzothiazole (BTA-2), diThT-C5, 6-iodo-2-(4'-dimethylamino-)phenyl-imidazo[1,2-a]pyridine (IMPY), DCVJ, and derivatives thereof. More preferably, the dye is dimeric Thioflavin T, such as diThT-PEG2, Thioflavin T,

or DCVJ. Yet more preferably, the dye is dimeric Thioflavin T, such as diThT-PEG2, Thioflavin T. Most preferably, the dye is Thioflavin T.

The present invention relates to methods for assessing whether an individual has a neurodegenerative disorder, in particular before the onset of clinical symptoms. The individual is preferably a human. The individual may be at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, or at least 70 years of age. Preferably, the individual is at least 60, or at least 65 years of age. Most preferably, the individual is at least 60 years of age. Alternatively, the individual may be up to 30, up to 35, up to 40, up to 45, up to 50, up to 55, up to 60, up to 65, or up to 70 years of age. Preferably, the individual has not been diagnosed with a neurodegenerative disorder. In addition, the individual preferably displays no clinical symptoms associated with a neurodegenerative disorder. Where the method is for assessing whether an individual has Parkinson's disease, the individual preferably displays no clinical symptoms associated with Parkinson's disease. Where the method is for assessing whether an individual has Alzheimer's disease, the individual preferably displays no clinical symptoms associated with Alzheimer's disease.

Clinical symptoms of Parkinson's disease include motor and non-motor symptoms and are well known to the skilled practioner. Primary motor symptoms include bradykinesia (slowness of movement), rigidity, resting tremor (for example in the hand, foot, jaw or face), or postural instability. Secondary motor symptoms include stooped posture, dystonia, fatigue, impaired fine motor dexterity and motor coordination, impaired gross motor coordination, poverty of movement, akathisia, speech problems, loss of facial expression, micrographia, difficulty swallowing, sexual dysfunction and drooling. Non-motor symptoms of Parkinson's disease include pain, sleep disturbances, hyposmia, constipation, skin problems, depression, fear or anxiety, memory difficulties and slowed thinking, urinary problems, fatigue and aching, loss of energy, compulsive behaviour and cramping. These symptoms are described in detail in WO2011/033252. The individual may not display any primary motor symptoms of Parkinson's disease. The individual may also not display any secondary motor symptoms of Parkinson's disease.

Clinical symptoms of Alzheimer's disease are also well-known in the art and include forgetfulness, confusion, depression, social withdrawal, mood swings, distrust in others, irritability and aggressiveness, changes in sleeping habits, wandering, loss of inhibitions, and delusions.

The individual may have been determined to be at risk of developing a neurodegenerative disorder, such as Parkinson's disease or Alzheimer's disease. For example, the individual may have been determined to have a mutation or polymorphism in a gene or locus associated with a neurodegenerative disorder. In addition, or alternatively, the individual may have a family history of a neurodegenerative disorder, such as a first degree relative with a neurodegenerative disorder. Where the neurodegenerative disorder is Parkinson's disease, the individual may have been determined to have a mutations or polymorphism in the SNCA gene, which encodes alpha-synuclein.

A neurodegenerative disorder, as referred to herein, may be characterised by an elevated number of, or larger, β-sheet containing oligomers in the CSF or peripheral blood plasma of an individual who has the neurodegenerative disorder compared with the number, or size, of such β-sheet containing oligomers in the CSF or peripheral blood plasma of a healthy individual. For example, a neurodegenerative disorder, may be characterised by an elevated number of, or larger, of β-sheet containing oligomers comprising, or consisting of, alpha-synuclein, amyloid-beta, and/or tau in the CSF or peripheral blood plasma of an individual who has the neurodegenerative disorder compared with the number, or size, of β-sheet containing oligomers of alpha-synuclein in the CSF or peripheral blood plasma of a healthy individual.

The neurodegenerative disorder is preferably characterised by protein aggregation. Protein aggregation is known to underlie a number of neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, Huntington's disease, dementia with Lewy bodies (DLB), pure autonomic failure (PAF), multiple system atrophy (MSA), Hallervorden-Spatz disease, frontal temporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), progressive supranuclear palsy, Pick's disease, corticobasal degeneration, transmissible spongiform encephalopathy, Amyotrophic lateral sclerosis, and amyloidosis.

A neurodegenerative disorder, as referred to herein, may be synucleinopathy. Synucleinopathies are a diverse group of neurodegenerative proteinopathies that share common pathological lesions composed of aggregates of conformational and posttranslational modifications of alpha-synuclein. Synucleinopathies include Parkinson's disease, dementia with Lewy bodies (DLB), pure autonomic failure (PAF), multiple system atrophy (MSA), and Hallervorden-Spatz disease.

Alternatively, a neurodegenerative disorder, as referred to herein, may be a tauopathy. Tauopathies are associated with the pathological aggregation of tau protein in the brain. Tauopathies include Alzheimer's disease, frontal temporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), progressive supranuclear palsy, Pick's disease, and corticobasal degeneration.

As a further alternative, the neurodegenerative disorder may be Huntington's disease, transmissible spongiform encephalopathy (such as Creutzfeldt-Jakob Disease, or variant Creutzfeldt-Jakob Disease), or amyotrophic lateral sclerosis.

Preferably, the neurodegenerative disorder is Parkinson's disease or Alzheimer's disease. Most preferably, the neurodegenerative disorder is Parkinson's disease.

In order to assess whether an individual has a particular neurodegenerative disorder, such as Parkinson's disease or Alzheimer's disease, for example, the method may further comprise subjecting an individual determined to have a neurodegenerative disorder using a method of the present invention to a further cognitive or genetic test to determine the neurodegenerative disorder present.

The present invention also relates to a method of delaying or preventing the onset of clinical symptoms associated with a neurodegenerative disorder, the method comprising assessing whether or not an individual has a neurodegenerative disorder using a method as described herein, and administering to an individual assessed as having a neurodegenerative disorder using said method a therapeutically effective amount of an agent that delays or prevents the onset of clinical symptoms associated with said neurodegenerative disorder.

The present invention also provides an agent for use in a method of delaying or preventing the onset of clinical symptoms associated with a neurodegenerative disorder, the method comprising:

(i) assessing whether or not an individual has a neurodegenerative disorder using a method as described herein; and

(ii) administering to an individual assessed as having a neurodegenerative disorder in (i) a therapeutically effective amount of an agent that delays or prevents the onset of clinical symptoms associated with said neurodegenerative disorder.

The agent which delays or prevents the onset of clinical symptoms associated with a neurodegenerative disorder may be an agent which directly or indirectly reduces the number and/or size of β-sheet containing amyloidogenic oligomers. For example, the agent which delays or prevents the onset of clinical symptoms associated with a neurodegenerative disorder may be an agent which directly or indirectly inhibits the formation of β-sheet containing amyloidogenic oligomers, and/or directly or indirectly induces the (full or partial) degradation of β-sheet containing amyloidogenic oligomers, and/or inhibits the degradation of β-sheet containing amyloid fibrils into β-sheet containing amyloidogenic oligomers. For example, the agent may directly or indirectly inhibit the formation of β-sheet containing oligomers comprising, or consisting of, alpha-synuclein, amyloid-beta, and/or tau, and/or directly or indirectly induce the degradation of β-sheet containing oligomers comprising, or consisting of, alpha-synuclein, amyloid-beta, and/or tau, and/or inhibit the degradation of β-sheet containing amyloid fibrils comprising, or consisting of, alpha-synuclein, amyloid-beta, and/or tau into β-sheet containing amyloidogenic oligomers comprising, or consisting of, alpha-synuclein, amyloid-beta, and/or tau. Preferably, the agent directly or indirectly inhibits the formation of β-sheet containing oligomers of alpha-synuclein, and/or directly or indirectly induces the degradation of β-sheet containing oligomers of alpha-synuclein, and/or inhibit the degradation of β-sheet containing amyloid fibrils of alpha-synuclein into β-sheet containing amyloidogenic oligomers of alpha-synuclein. In this case, the neurodegenerative disorder is preferably a synucleinopathy, most preferably Parkinson's disease. Alternatively, the agent may directly or indirectly inhibit the formation of β-sheet containing oligomers of amyloid-beta and/or tau, and/or directly or indirectly induce the degradation of β-sheet containing oligomers of amyloid-beta and/or tau, and/or inhibit the degradation of β-sheet containing amyloid fibrils of amyloid-beta and/or tau into β-sheet containing amyloidogenic oligomers of amyloid-beta and/or tau. In this case, the neurodegenerative disorder is preferably Alzheimer's disease.

For example, polyphenols have been shown to be capable of disaggregating oligomeric structures, and inhibit formation of oligomers *in vitro*. One such compound, curcumin, has also been demonstrated to bind to monomeric alpha-synuclein with high affinity, and reduces its propensity to aggregate and prevent formation of oligomers and fibrils. Thus, an agent which inhibits the formation of β-sheet containing amyloidogenic oligomers, and/or directly or indirectly induces the degradation of β-sheet containing amyloidogenic oligomers may be a polyphenol, such as curcumin.

With regard to alpha-synuclein, several post-translational modifications have been described which alter the propensity of the protein to aggregate. These include phosphorylation of the serine residue at position 129, which is regulated by protein kinases and phosphatases. Therefore kinase inhibitors could reduce the phosphorylation state of the protein and thereby alter the formation of oligomers. Large kinase inhibitor screens have already been developed for their use in the treatment of cancer, and therefore a library of potentially useful compounds is readily available to test such a strategy. Thus, an agent which inhibits the formation of β-sheet containing amyloidogenic oligomers, such as β-sheet containing oligomers of alpha-synuclein, may be a kinase inhibitor.

In addition, it has been shown that cuminaldehyde (4-isopropylbenzaldehyde) can inhibit the formation of alpha-synuclein fibrils. Thus, an agent which inhibits the formation of β-sheet containing amyloidogenic oligomers, such as β-sheet containing oligomers of alpha-synuclein, may be cuminaldehyde.

Furthermore, monoclonal antibodies can be generated towards specific protein conformations, such as β-sheet containing amyloidogenic oligomers, including β-sheet containing amyloidogenic oligomers of alpha-synuclein, and have been shown to be able to internalised by cells and reduce the intracellular number of oligomers in culture. Phage display technology can also be used to isolate antibodies, or antigen-binding fragments thereof, that recognise specific β-sheet containing amyloidogenic oligomers. Immunisation using antibodies, or antigen-binding fragments thereof, capable of binding to β-sheet containing amyloidogenic oligomers may be used to treat neurodegenerative disorders, such as PD. Thus, an agent which directly or indirectly induces the degradation of β-sheet containing amyloidogenic oligomers may be an antibody or nanobody which specifically binds to β-sheet containing amyloidogenic oligomers, such as β-sheet containing oligomers of alpha-synuclein.

Vaccinations of recombinant human alpha-synuclein (or injection of a monoclonal anti-alpha-synuclein antibody) in transgenic mice has been shown to reduce the number of alpha-synuclein oligomers. Translation of such preclinical studies to clinical trials is underway with phase 1 studies. An agent which directly or indirectly reduces the number of β-sheet containing amyloidogenic oligomers may therefore be a vaccine comprising β-sheet containing amyloidogenic oligomers, such as β-sheet containing oligomers of alpha-synuclein, or an antibody specific for β-sheet containing amyloidogenic oligomers, such as β-sheet containing oligomers of alpha-synuclein.

A different strategy to remove β-sheet containing amyloidogenic oligomers is to promote the body's own defence systems against misfolded proteins. The systems that regulate clearance of oligomers are the chaperones (such as hsp70 and CHIP) that regulate protein folding and refolding, the ubiquitin-proteasome system (UPS), and the autophagy-lysosomal pathway (ALP) (which eliminate harmful proteins). Small molecule inhibitors of hsp70, or upregulators of the UPS or ALP may be beneficial in the removal of β-sheet containing amyloidogenic oligomers in disease. Thus, an agent which directly or indirectly induces the degradation of β-sheet containing amyloidogenic oligomers, such as β-sheet containing oligomers of alpha-synuclein, may be a small molecule inhibitor of hsp70, or an upregulator of the UPS or ALP.

Alternatively, an agent which delays or prevents the onset of clinical symptoms associated with a neurodegenerative disorder may be an agent that binds to β-sheet containing amyloidogenic oligomers. The agent preferably inhibits one or more activities of β-sheet containing amyloidogenic oligomers associated with the development of a neurodegenerative disorder. For example, the agent may inhibit interactions, in particular damaging interactions, between β-sheet containing amyloidogenic oligomers and cells. The agent may be an antibody, such as a neutralizing antibody. Methods for making antibodies, including neutralizing antibodies, which bind to an antigen of interest are well-known in the art.Further provided is a method of treating a neurodegenerative disorder, the method comprising assessing whether or not an individual has a neurodegenerative disorder using a method as described herein, and administering to an individual assessed as having a neurodegenerative disorder using said method a therapeutically effective amount of an agent that treats said neurodegenerative disorder.

The present invention also provides an agent for use in a method of treating a neurodegenerative disorder, the method comprising:

(i) assessing whether or not an individual has a neurodegenerative disorder using a method of the present invention; and

(ii) administering to an individual assessed as having a neurodegenerative disorder in (i) a therapeutically effective amount of an agent that treats said neurodegenerative disorder.

Agents for the treatment of neurodegenerative disorders, such as Parkinson's disease or Alzheimer's disease are known. In the case of Parkinson's disease, the agent may be levodopa (optionally in combination with a dopa decarboxylase inhibitor or COMT inhibitor,

such as entacapone, nitecapone, and/or tolcapone), a dopamine agonist (such as bromocriptine, pergolide, pramipexole, ropinirole, piribedil, cabergoline, apomorphine, and/or lisuride) and/or a MAO-B inhibitor (such as selegiline and/or rasagiline). In the case of Alzheimer's disease, the agent may be a cholinesterase inhibitor, such as donepezil, rivastigmine, or galantamine. Alternatively, the agent may be memantine. The agent may be a combination of a memantine and a cholinesterase inhibitor.

Agents will usually be administered in the form of a pharmaceutical composition, which may comprise at least one component in addition to the agent. The pharmaceutical composition may comprise, in addition to the agent, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration.

Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilizers, buffers, antioxidants and/or other additives may be employed, as required. Many methods for the preparation of pharmaceutical formulations are known to those skilled in the art. See e.g. Robinson ed., Sustained and Controlled Release Drug Delivery Systems, Marcel Dekker, Inc., New York, 1978.

An agent as described herein may be administered alone or in combination with another agent or agents, concurrently or sequentially or as a combined preparation with another agent or agents, dependent upon the condition to be treated. For example, an agent for use in the invention may be used in combination with an existing therapeutic agent for the disease to be treated.

Administration may be in a "therapeutically effective amount", this being sufficient to show benefit to a patient. Such benefit may be delay or prevention of the onset of clinical symptoms associated with a neurodegenerative disorder, or amelioration of at least one symptom associated with a neurodegenerative disorder. "Treatment" of a neurodegenerative disorder may therefore refer to amelioration of at least one symptom associated with a neurodegenerative disorder. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of the neurodegenerative disorder being treated, the particular patient being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the composition, the type of conjugate, the method of administration, the scheduling of administration and other factors known to medical practitioners. Prescription of agents, e.g. decisions on dosage etc., is within the responsibility of general practitioners and other medical doctors. A therapeutically effective amount or suitable dose of a particular agent can be determined by comparing its *in vitro* activity and *in vivo activity* in an animal model. Methods for extrapolation of effective dosages in mice and other test animals to humans are known. The precise dose will depend upon a number of factors, including the precise nature of the agent. Treatments may be repeated at daily, twice-weekly, weekly or monthly intervals, at the discretion of the physician.

Also provided are methods for identifying agents capable of directly or indirectly inhibiting the formation of β-sheet containing amyloidogenic oligomers, inducing the degradation of β-sheet containing amyloidogenic oligomers, and/or inhibiting the degradation of β-sheet containing amyloid fibrils into β-sheet containing amyloidogenic oligomers. The β-sheet containing amyloidogenic oligomers in this context may be β-sheet containing amyloidogenic oligomers consisting of alpha-synuclein, amyloid-beta and/or tau, or consisting of amyloid-beta and/or tau. The β -sheet containing amyloidogenic oligomers may be β-sheet containing amyloidogenic oligomers consisting of alpha-synuclein. Alternatively, the β -sheet containing amyloidogenic oligomers may be β-sheet containing amyloidogenic oligomers consisting of amyloid-beta. As a further alternative, the β -sheet containing amyloidogenic oligomers may be β-sheet containing amyloidogenic oligomers consisting of tau. As a yet further alternative, the β -sheet containing amyloidogenic oligomers may be β-sheet containing amyloidogenic oligomers consisting of prion protein (PrP^{sc}). Preferably, the β -sheet containing amyloidogenic oligomers are β-sheet containing amyloidogenic oligomers consisting of alpha-synuclein.

Methods for identifying agents capable of directly or indirectly inhibiting the formation of β-sheet containing amyloidogenic oligomers include *in vitro* methods and *in vivo* methods employing the use of a non-human animal that is a model of a neurodegenerative disorder. In the case of methods employing an animal model,

The non-human animal may be any animal which can serve as a model of neurodegenerative disorder. Many non-human animal models of neurodegenerative disorders, such as Parkinson's disease, are known in the art and can be employed in the present methods. The animal may be a non-human animal which exhibits expression, or exhibits increased expression, of a protein or protein fragment, e.g. a human protein or human protein fragment, capable of forming β-sheet containing amyloidogenic oligomers. Preferably, the animal exhibits expression, or increased expression, of the protein in a biofluid (e.g. the CSF and/or peripheral blood plasma) of the animal.

For example, the animal may be a transgenic non-human animal having a genome which has been altered to express a protein or protein fragment, preferably a human protein or human protein fragment, capable of forming β-sheet containing amyloidogenic oligomers. Alternatively, the animal may be a non-human animal which has been treated with a substance resulting in the expression, or increased expression, of a protein or protein fragment, e.g. a human protein or human protein fragment, capable of forming β-sheet containing amyloidogenic oligomers. The substance may be a neurotoxin, or a virus expressing a protein or protein fragment, preferably a human protein or human protein fragment, capable of forming β-sheet containing amyloidogenic oligomers. An increased expression in this context preferably refers to an increased expression of the protein relative to the expression level of the protein in a control animal which has not been treated with the substance. When comparing the expression level of a protein, expression of the protein in the same biofluid, e.g. the CSF or peripheral blood plasma, obtained from the respective animals is preferably compared.

The non-human animal may be a non-human primate, such as rhesus macaque or chimpanzee, chicken, cat, dog, or rodent, such as mouse or rat. Preferably, the non-human animal is a rodent, more preferably a rat or mouse. Most preferably the non-human animal is a mouse.

In the context of these methods, the β-sheet containing amyloidogenic oligomers, or β-sheet containing amyloid fibrils, may be labelled with a second fluorescent dye, e.g. an organic

dye, such as Alexa Fluor 647 (AF647). In this case, the fluorescence intensity of the second fluorescent dye may be compared to the fluorescence intensity of the fluorescent dye which exhibits a change in one or more photophysical properties upon binding to a β-sheet containing amyloidogenic oligomer employed in the method, to determine the number and/or size of the β-sheet containing amyloidogenic oligomers present.

In a further aspect, the present invention relates to an *in vitro* method for determining the number and/or size of β-sheet containing amyloidogenic oligomers in sample, such as a cerebrospinal fluid (CSF) or a peripheral blood plasma sample, using a method as described herein.

Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure.

Other aspects and embodiments of the invention provide the aspects and embodiments described above with the term "comprising" replaced by the term "consisting of" and the aspects and embodiments described above with the term "comprising" replaced by the term "consisting essentially of".

It is to be understood that the application discloses all combinations of any of the above aspects and embodiments described above with each other, unless the context demands otherwise. Similarly, the application discloses all combinations of the preferred and/or optional features either singly or together with any of the other aspects, unless the context demands otherwise.

Modifications of the above embodiments, further embodiments and modifications thereof will be apparent to the skilled person on reading this disclosure, and as such these are within the scope of the present invention.

All documents and sequence database entries mentioned in this specification are incorporated herein by reference in their entirety for all purposes.

"and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein.

Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figures described above.

Examples

Example 1 – Preparation of labelled alpha-synuclein

The A90C variant of monomeric alpha-synuclein was purified from Escherichia coli as described previously (Hoyer *et al.*, 2004; Cremades *et al.*, 2012). The single cysteine was labelled with maleimide-modified Alexa Fluor 647 (AF647) as previously reported (Thirunavukkuarasu *et al.*, 2008). The excess dye was removed by passing the labelled protein through a P10 desalting column containing Sephadex G25 matrix (GE Healthcare, Waukesha, WI, USA). The protein was concentrated using Amicon Ultra Centricons (Millipore, Billerica, MA, USA), divided into aliquots, before being flash-frozen and stored at -80°C. Each aliquot was only thawed once prior to use.

Example 2 – Aggregation of labelled alpha-synuclein

For the aggregation reactions, a 70 μM solution of AF647 labelled alpha-synuclein in 25 mM Tris buffer (pH 7.4) and 0.1M NaCl (with 0.01% NaN_3 to prevent bacterial growth during the experiments) was incubated in the dark at 37°C, with constant agitation at 200 rpm for 5 days, during which time aliquots were taken.

Example 3 – Preparation of an enriched oligomer solution of alpha-synuclein

Briefly, a solution of monomeric alpha-synuclein at ca. 800 μM (12 mg/ml) was incubated in PBS at 37°C without agitation for 20-24 h. The solution was then passed through a 0.22 μm cut-off filter to remove any particles of dust and/or big protein aggregates that may have been present. The monomeric fraction of the solution (> 95%) was removed from the oligomers of alpha-synuclein by passing the solution through a series of 100 kDa cut-off filters. The concentration of the final oligomeric solution was estimated from the absorbance at 275 nm, using an extinction coefficient of 5,600 M^{-1} cm^{-1}. The concentration was found to be in the range of 1-100 μM.

Example 4 – Preparation of Thioflavin T (ThT) solution

ThT stock solutions were prepared by diluting ThT (Sigma Aldrich) into neat ethanol (Sigma Aldrich) to give a final concentration of ~1 M. Following this, a hundred-fold dilution into pre-filtered PBS (0.02 µm syringe filter, Whatman) was performed to give a ~100 µM stock solution of ThT. The final concentration was determined from the absorbance at 412 nm, using an extinction coefficient of 36,000 M^{-1} cm^{-1}. The stock solution was stored in the dark at 4°C, and was only used for a maximum of one week after preparation.

Example 5 – Single-molecule imaging using ThT and total internal reflection fluorescence (TIRF) microscopy

Preparation of slides for single-molecule analysis

Borosilicate glass coverslips (VWR international, 20×20 mm, 63 1-0122, lot 4140881) were cleaned using an argon plasma cleaner (PDC-002, Harrick Plasma) for 1 hour to remove any fluorescent residues. 9×9 mm Frame-Seal slide chambers (Bio-rad, Hercules, CA, USA) were affixed to the glass, and 50 µL of Poly-L-lysine (Sigma-Aldrich) was added to the coverslides on the inside of the chamber and incubated for at least thirty minutes, before being washed with filtered PBS Buffer. Prior to use, each batch of coverslides was tested for artefacts by analysing a solution of 5 µM ThT only. We found that the Poly-L-lysine solution degraded over time, leading to fluorescent artefacts and hence false positives, so solutions of this were only used for a maximum of 4 hours before being discarded.

Single-molecule imaging

Single-molecule imaging was performed using a home-built total internal reflection fluorescence (TIRF) microscope or a home-built confocal microscope.

Imaging using a TIRF microscope restricts detectable fluorescence signal to within ~200 nm from the sample slide. For imaging of Alexa Fluor 647 labelled alpha-synuclein in the presence of ThT, the output from two lasers operating at 405 nm (Oxxius LaserBoxx) and 641 nm (Coherent Cube) were aligned and directed parallel to the optical axis at the edge of a 1.49 numerical aperture (NA) 60X TIRF objective (N2709400, APON60XO TIRF, Olympus) mounted on an Olympus IX-71 microscope. The intensity/power of the laser operating at 405 nm was 150-200 W/cm^2, while the power/intensity of the laser operating at 641 nm was

10-50 W/cm^2. Fluorescence was collected by the same objective was separated from the returning TIR beam by a dichroic (Di01-R4-5/488/561/635, Semrock), and passed through appropriate filters (BLP01-488R and BLP01-635R, Semrock). The images were recorded on an EMCCD camera (Evolve 512, Photometrics) operating in frame transfer mode. For each data-set, 3 x 3 image grids were measured in three different regions of the coverslide. The distance between the 9 images measured in each grid was set to 350 µm, and was automated to prevent user bias. Images were recorded at 33 frames s^{-1} for 150 frames firstly from the red channel (Alexa 647 emission) with 641 nm illumination, followed by 150 frames in the blue channel (ThT emission) with 405 nm illumination. For unlabelled oligomers, either from the enriched preparation, or those present in CSF, the sample was only excited with 405 nm illumination, and images were recorded from the short wavelength channel only.

Single-molecule fluorescent burst counting was performed using a home-built confocal microscope (Horrocks M. H., 2011). A 488 nm laser beam (Spectra Physics, Cyan CDRH, Newport Corporation, Oxford, UK) was first attenuated using neutral density filters, and passed through a spatial filter to ensure the beam was TEM00, before being passed through the back-port of an inverted microscope (Nikon Ti-U). A dichroic mirror (Semrock, Di01-R405/488/594) reflected the light through an oil-immersion objective (Nikon CFI Plan Apochromat VC 60X Oil N2 NA 1.4, W.D 0.13 mm) which focused it to a diffraction limited confocal spot within the sample being studied. The emitted fluorescence was collected by the same objective and passes through the same dichroic, before being focused by a tube lens within the microscope body through a 50 µm pinhole (Thorlabs). The light was focused by a lens (Plano apo convex, focal length = 50 mm, Thorlabs) through a long-pass filter (BLP01-488R) onto the Avalanche Photodiode (APD) detector. The outputs from the APD were connected to a custom-programmed field-programmable gate array, FPGA (Colexica), which counted the signals and combined them into time bins which were selected according to the expected residence time of molecules traveling through the confocal volume.

For confocal microscopy, the alpha-synuclein sample was diluted to 1 µm into 500 nM ThT and flowed through a microfluidic channel (100 x 25 µm diameter) at 2 cm/s. The confocal volume was focused into the center of the channel, and fluorescence was measured for 5 minutes. The laser intensity was 2 mW at the back-port of the instrument.

Example 6 - Data analysis

For the labelled alpha-synuclein, each data-set consisted of 27 image stacks of 150 frames from both the blue and red channels, measured in different regions of the cover-slide to reduce area-to-area variation. These resulting images were automatically analysed using computer software which counts the total number of visible diffraction-limited spots above background, although in principle any algorithm capable of discriminating these could be used, data analysis was performed using ImageJ (NIH). The stacks was first averaged over the 150 frames for each channel and the "Find Maxima" command (based on a plugin contributed by Michael Schmid) was used to detect spots present in the averaged blue channel, and the intensities of the detected spots were then quantified in both the blue and red channels. An empirically determined standard noise tolerance level was set to differentiate the spots from the background (maxima were ignored if they did not stand out from the surroundings by more than 1000 counts). For unlabelled samples, stacks only in the blue channel were recorded and analysed.

Example 7 - Detection of ThT labelled oligomers

Although free ThT is fluorescent, the quantum yield of its fluorescence is much lower than when bound to extended β-sheet regions of amyloid species ($\Phi_{fluorescence}$ = 0.0001 in water at room temperature, compared to 0.43 when bound to insulin fibrils [Sulatskaya *et al.*, 2010]). For this reason, it is possible to use ThT at very high (micromolar) single-molecule concentrations without the autofluorescence being so high that it prevents detection of single-molecules of ThT-bound oligomers. In order to determine the optimum ThT concentration to use for oligomer detection, a sample containing oligomeric alpha-synuclein was imaged at a range of ThT concentrations and the average number and intensity of the spots detected at different ThT concentrations was determined. As the concentration of ThT increased, the number and intensity of spots detected increased, since more ThT was available to bind to the oligomers. The maximum number of spots was detected at a concentration of 5 μM ThT. At concentrations above 5 μM ThT, the detection efficiency decreased, since the background signal increased more than the signal from single oligomers, leading to a decrease in the signal-to-noise ratio. Consequently, a ThT concentration of 5 μM was used for all experiments.

ThT binds to β-sheet structures with a low micromolar affinity, and exchange is rapid; this was exemplified by the fluorescence intensity of the oligomers, which did not decrease over

time, even at high laser powers, meaning that the rate of exchange must be greater than the rate of photobleaching. This allows oligomers to be imaged for prolonged periods of time which allowed for greater discrimination between real oligomers (which do not appear to photobleach as the rate of dye exchange is higher) and other fluorescent impurities (which do photobleach). This effect was taken advantage of during the data-collection procedure; over the 5 seconds that the stacks were recorded, the fluorescent impurities were bleached, and so by averaging over the whole stack, the signal-to-background ratio was higher. In contrast the AF647 labels were bleached over time as evidenced by a stepped decrease in fluorescence intensity

Example 8 – Sensitivity of single-molecule imaging

To determine the range of alpha-synuclein oligomer concentrations that were detectable, experiments were performed on a number of concentrations of enriched oligomers, prepared as described in Example 3, diluted into either PBS or CSF from a control sample containing very few oligomers to begin with. The oligomer preparations were analysed using the TIRF method employing ThT described in Example 5.

At concentrations spanning 1-100 nM, there was a linear relationship between the concentration of oligomers and the number of detected events. At lower concentrations (~200 pM), the number of spots counted (5.5 ± 2.3 for dilution into PBS, and 11.2 ± 3.1 for dilution into CSF) was similar to that from background alone (2.3 ± 1.2 and 10.8 ± 2.1 for dilution into PBS and CSF, respectively), and so detecting oligomers became problematic. The limit of blank (LoB) is the highest apparent number of species expected to be found when replicates of a sample containing no analyte are detected, and is given by (Armbuster *et al.*, 2008):

$$LoB = mean\ blank + 1.645 \times (S.D.\ blank)$$

The limit of detection (LoD) is determined by utilising both the measured LoB and test replicates of a sample known to contain a low concentration of analyte, and is defined as:

$$LoD = LoB + 1.645 \times (S.D.\ of\ low\ concentration\ sample)$$

The LoBs were determined to be 4.4 and 7.1, and the LoDs were calculated as 8.1 and 12.1 for oligomers diluted into PBS and CSF, respectively.

At higher concentrations (~ 1 μM), the number of spots in the field of view became too high, and counting individual spots was not possible.

Example 9 – Detection of cytotoxic β-sheet containing oligomers of alpha-synuclein using single-molecule imaging

Cytotoxic oligomers, which are formed when labelled monomeric alpha-synuclein is left to incubate under aggregating conditions have been previously observed and characterized (Cremades *et al.*, 2012). In that study, globular non-toxic oligomers (type A) were first formed. These could then convert to form toxic, presumably β-sheet containing oligomers (type B), and finally fibrils.

Alexa Fluor 647 (AF647) labelled alpha-synuclein was prepared as set out in Example 1 and incubated under conditions favouring aggregation as set out in Example 2. Samples taken at different times during the aggregation process were analysed using single-molecule imaging with a TIRF microscope and confocal microscope as described in Example 5. By using alpha-synuclein labelled with AF647, the present inventors were able to detect not only the ThT active species (**Figure 1B**), but also the monomeric and globular oligomers from AF647 fluorescence (**Figure 1C**) using TIRF. During the first day of the oligomerization process, the protein sample was mainly monomeric, with some amorphous oligomers; both species lack the β-sheet structure needed for ThT binding, meaning only fluorescence from AF647 was observed. After one day, some of the amorphous oligomers had undergone a structural conversion (half-life is ~35 hours [Cremades *et al.* 2012]) to form β-sheet containing oligomers that bound ThT; these were observed as brighter diffraction-limited spots coincident in both the ThT and AF647 channels. Both the brightness and number of spots increased, until after three days, fibrils were observed and the number of oligomers decreased. **Figure 1E** shows the time-progression of the number of ThT active oligomers, and this correlates well with the high fluorescence resonance energy transfer (FRET), cytotoxic oligomers identified previously using labelled alpha-synuclein, suggesting that these do indeed correspond to β-sheet rich oligomers, and that single-molecule total internal fluorescence (TIRF) microscopy correlates well with previous established techniques used to study oligomerization kinetics, two colour coincidence detection (TCCD). **Figure 1E** shows the oligomer counts from TIRF images taken of an aggregation of 70 μM alpha-synuclein,

diluted to 700 nM into 5 μM ThT. Mean from three aggregations, and error bars are standard deviation.

Figure 1D shows the time-progression of the number of ThT active oligomers as determined using single-molecule confocal microscopy, and again this correlates well with the high fluorescence resonance energy transfer (FRET), cytotoxic oligomers identified previously using labelled alpha-synuclein, further supporting the conclusion that these do indeed correspond to β-sheet rich oligomers, and that single-molecule confocal microscopy also correlates well with previous established techniques used to study oligomerization kinetics, two colour coincidence detection (TCCD).

Example 10 – Characterisation of an enriched oligomer preparation

In order to show that the species detected using single-molecule imaging as described in Example 5 are oligomeric, the technique was applied to a sample of an enriched oligomer solution of alpha-synuclein generated using a protocol that is able to form solutions of >95% alpha-synuclein oligomer as described in Example 3 .

The size and structure of the oligomers present in the sample was determined using multiple biophysical techniques including: atomic force microscopy (AFM), circular dichroism (CD) and dynamic light scattering (DLS), before being characterised by our new single-molecule imaging technique (**Figure 2**).

Atomic force microscopy (AFM): a PicoPlus AFM with a PicoSPM II controller from Molecular Imaging was used for imaging the alpha-synuclein oligomers. Images were acquired at room temperature in air using the AC Mode with NSC36/no Al cantilevers (Mikromasch, force constant varying from 0.6 to 1.75 N/m). For imaging, an aliquot of the alpha-synuclein species was deposited onto freshly cleaved mica, incubated for 10 min, followed by washes with deionised water and dried under a stream of nitrogen.

Circular dichroism (CD): far-UV CD spectra of the alpha-synuclein oligomers were acquired in PBS at 20°C. Proteins were diluted to a final concentration of ~10 μM and the spectra were acquired using a 1 mm path length cuvette and a J-810 Jasco spectropolarimeter (Tokyo, Japan), equipped with a thermostatic cell holder.

Dynamic light scattering (DLS): the intensity-weighted mean hydrodynamic diameter with polydispersity index, PdI (a parameter used to describe the width of the size distribution), and the diameter number distribution profile of oligomeric alpha-synuclein (5 μM) in PBS at 25°C were determined by DLS using a Zetasizer Nano ZS (Malvern Instruments) at a back scattering angle of 173°.

The DLS showed that the oligomers had a hydrodynamic radius (peaks at ~13 nm) in between that of monomer (~4 nm) and fibril (~2000 nm), and the CD showed an intermediate spectrum between the random coil-like monomer and the β-sheet rich fibrillar species. The AFM showed only spherical species consistent with oligomers and not fibrils (see **Figure 2B, C and D**). Specifically, DLS showed that the oligomers had a diameter between 10 and 50nm (**Figure 2C**).

As expected, diffraction limited spots from single-molecule imaging using ThT fluorescence were observed from the samples, therefore highlighting that this technique is both capable of detecting oligomeric species and differentiating them from larger fibrillar structures (see **Figure 2A**).

Example 11 – Detection of β-sheet oligomers in human cerebral spinal fluid (CSF) samples using ThT and diThT-PEG

With the methodology established on oligomers formed *in vitro*, the inventors attempted to detect β-sheet containing oligomers in human cerebral spinal fluid (CSF) samples using single-molecule imaging as described in Example 5. CSF samples were collected from 18 patients with clinically defined Parkinson's disease (PD) (aged 51-85 years, mean ± S.D = 66 ± 8) and 20 normal healthy individuals (aged 46-71 years, mean ± S.D = 61 ± 9). The patients were diagnosed according to the UK PD Society Brain Bank Criteria. Ethical approval for collection was obtained for all samples. The samples were stored at -80°C before analysis, and repeat freeze/thaw cycles were avoided.

Using 5 μM ThT and single-molecule imaging as described in Example 5, it was possible to observe β-sheet containing oligomers in 36 CSF samples from both PD patients and healthy controls (**Figure 3C**). Unlike ELISA, ThT binding is sensitive to any β-sheet containing oligomers, including β-sheet containing oligomers of alpha-synuclein, which have previously been identified as being cytotoxic (Cremades *et al.*, 2012). The β-sheet containing oligomers once again appeared as diffraction limited spots, with intensity distributions similar to those

observed for the cytotoxic β-sheet containing alpha-synuclein oligomers formed *in vitro*. **Table 1** below and **Figure 3A-D** show the results from the analysis of the CSF samples. A statistically highly significant difference between the oligomer counts between CSF samples from PD patients and healthy controls (HC) was observed (there was a mean of -0.007 [95% confidence interval (CI): -0.01 to -0.004] fewer counts in HC compared to PD patients, $p<0.0001$) (**Figure 3C**), therefore suggesting that the number of β-sheet containing amyloidogenic oligomers present in a sample can be used assess whether an individual has a neurodegenerative disorder, such as Parkinson's disease.

Similarly, a statistically significant difference in the photon count intensity distribution histograms from two representative CSF samples from a PD patient and a healthy controls (HC) was observed (**Figure 3A**). It is thought that the photon count is proportional to the size of the oligomer. These results therefore suggest that the size of the β-sheet containing amyloidogenic oligomers present in a sample can be used to assess whether an individual has a neurodegenerative disorder, such as Parkinson's disease.

Table 1. Clinical details of patients with PD, giving the number of oligomers (mean from three sets of 3x3 grids ± S.D.) and oligomer brightness (mean from three sets of 3x3 grids ± S.D.). H-Y - Hoehn & Yahr grade.

Case	Age	Duration (years)	H-Y Score	Number of oligomers (counts/μm²)	Oligomer brightness (photons)
1	68	20	4	0.0144 ± 0.0078	3537 ± 452
2	75	14	4	0.0148 ± 0.0085	3295 ± 556
3	58	5.5	2	0.0129 ± 0.0048	4692 ± 2086
4	69	11	3	0.0100 ± 0.0045	3260 ± 591
5	53	5	1	0.0237 ± 0.0047	5032 ± 437
6	60	6	2	0.0148 ± 0.0061	3552 ± 355
7	62	15	4	0.0160 ± 0.0031	5711 ± 490
8	51	5	1	0.0098 ± 0.0017	2423 ± 1274
9	57	1.5	2	0.0112 ± 0.0026	2980 ± 337
10	71	15	2	0.0137 ± 0.0021	3193 ± 283
11	74	18	3	0.0055 ± 0.0012	3101 ± 171
12	65	15	3	0.0177 ± 0.0069	4165 ± 1083
13	63	14	3	0.0189 ± 0.0034	4879 ± 607
14	63	13	2	0.0088 ± 0.0012	2644 ± 358
15	72	17	5	0.0134 ± 0.0027	3493 ± 450
16	66	5	2	0.0085 ± 0.0009	3154 ± 338
17	68	5	2	0.0027 ± 0.0009	3802 ± 215
18	85	14	4	0.0169 ± 0.0012	3714 ± 504

Table 2. Clinical details of control subjects, giving the number of oligomers (mean from three sets of 3x3 grids ± S.D.) and oligomer brightness (mean from three sets of 3x3 grids ± S.D.).

Case	Age	Number of oligomers (counts/μm²)	Average oligomer brightness (photons)
19	69	0.0016 ± 0.0078	2793 ± 260
20	69	0.0022 ± 0.008469	3254 ± 835
21	67	0.0049 ± 0.0048	5863 ± 1139
22	64	0.0069 ± 0.0045	2874 ± 357
23	64	0.0055 ± 0.0047	2377 ± 443
24	63	0.0045 ± 0.0061	3149 ± 229
25	61	0.0174 ± 0.0031	4598 ± 722
26	76	0.0058 ± 0.0017	2502 ± 112
27	62	0.0038 ± 0.0026	2413 ± 217
28	71	0.0028 ± 0.0021	3447 ± 555
29	71	0.0124 ± 0.0012	3358 ± 375
30	64	0.0046 ± 0.0069	2937 ± 747
31	46	0.0064 ± 0.0034	3914 ± 1234
32	56	0.0044 ± 0.0012	2867 ± 335
33	64	0.0072 ± 0.0027	6470 ± 150
34	46	0.0026 ± 0.0009	3484 ± 76
35	45	0.0026 ± 0.0009	3376 ± 625
36	61	0.0043 ± 0.0012	3406 ± 134

Using single-molecule imaging as described in Example 5 but employing 1μM diThT-PEG rather than ThT, it was also possible to observe β-sheet containing oligomers in CSF samples from both PD patients and healthy controls (**Figure 3E**). Unlike ELISA, diThT-PEG binding is sensitive to any β-sheet containing oligomers, including β-sheet containing oligomers of alpha-synuclein, which have previously been identified as being cytotoxic (Cremades *et al.*, 2012). The β-sheet containing oligomers once again appeared as diffraction limited spots, with intensity distributions similar to those observed for the cytotoxic β-sheet containing alpha-synuclein oligomers formed *in vitro*. **Figure 3E** show the results from the analysis of 26 CSF samples. A statistically highly significant difference between the oligomer counts between CSF samples from PD patients and healthy controls (HC) was observed (there was a mean of 0.0092 spots/ um^2 [95% confidence interval (CI): 0.0061 to 0.0153 spots/um^2] fewer counts in HC compared to PD patients, $p<0.0001$) (Figure 3E), providing further evidence that the number of β-sheet containing amyloidogenic oligomers present in a sample can be used assess whether an individual has a neurodegenerative disorder, such as Parkinson's disease. However, despite the fact that diThT-PEG had a

higher affinity for β-sheet containing oligomers than ThT, the separation of PD patients and healthy controls based on the spot density observed using diThT-PEG (spot density corresponds to the number of oligomers detected) was less good than for ThT. Without wishing to be limited by theory, it is believed that diThT-PEG does not detect smaller β-sheet containing oligomers which can be detected using ThT, resulting in less separation between the PD patients and the healthy controls. Use of ThT is therefore preferred over the use of diThT-PEG.

The above results demonstrate that the single-molecule imaging method of the present invention can be used to detect unlabelled amyloidogenic oligomers containing β-sheet structure, *e.g.* alpha-synuclein oligomers, as well as to count and characterize such oligomers formed *in vivo*. The method of the invention has been used to show that CSF samples from patients with Parkinson's disease contain significantly more β-sheet containing oligomers compared to samples from healthy controls. In contrast to the ELISA technique, the method of the present invention allows all of the β-sheet oligomers present to be directly counted, as well as to determine their relative sizes via the fluorescence intensity, which is a unique advantage of the single-molecule detection method of the present invention. In addition to having potential diagnostic applications, the method of the invention may help determine the oligomeric species in humans that lead to the onset and progression of PD, and is expected to be applicable to other neurodegenerative diseases, such as Alzheimer's Disease.

Example 12 – Detection of β-sheet containing oligomers of alpha-synuclein using dimeric thioflavin-T

diThT-PEG2 was purchased from Peakdale synthesis, and stock solutions were prepared by diluting it into neat ethanol (Sigma Aldrich) to give a final concentration of ~1 M. Following this, a hundred-fold dilution into pre-filtered PBS (0.02 μm syringe filter, Whatman) was performed to give a ~100 μM stock solution of diThT-PEG. The final concentration was determined from the absorbance at 412 nm, using an extinction coefficient of 45,800 M^{-1} cm^{-1}. The stock solution was stored in the dark at 4°C, and was only used for a maximum of two weeks after preparation.

Wild type alpha-synuclein was incubated under conditions favouring aggregation as set out in Example 2. Samples containing oligomers were taken and diluted (to ~1 nM) into 5 μM diThT-PEG2, before being imaged with a TIRF microscope as described in Example 5. CSF

from a PD patient was diluted 1/10 into 5 μM di-ThT-PEG2 and imaged using TIRF microscopy.

Each data-set consisted of 27 image stacks of 150 frames measured in different regions of the cover-slide to reduce area-to-area variation. These resulting images were automatically analysed using computer software which counts the total number of visible diffraction-limited spots above background, although in principle any algorithm capable of discriminating these could be used, data analysis was performed using ImageJ (NIH). The stacks was first averaged over the 150 frames and the "Find Maxima" command (based on a plugin contributed by Michael Schmid) was used to detect spots present in the averaged blue channel, and the intensities of the detected spots were then quantified in this channel. An empirically determined standard noise tolerance level was set to differentiate the spots from the background (maxima were ignored if they did not stand out from the surroundings by more than 1000 counts). For unlabelled samples, stacks only in the blue channel were recorded and analysed.

As diThT-PEG2 binds with a higher affinity, and is brighter than ThT, then the oligomers appeared as brighter diffraction limited spots, as shown in **Figures 4C** and **D**, and is evident from the intensity distributions shown in **Figures 4E** and **F**.

Example 13 – Detection of β-sheet containing oligomers of alpha-synuclein by measuring fluorescence lifetime

Fluorescence lifetime was measured by diluting an aggregated sample of alpha-synuclein to 350 nM into 20 μM ThT, and measuring the lifetime on a commercial FLIM microscope (Leica TCS SP8 SMD) with excitation at 440 nm and detection at >500 nm. When bound to oligomers of alpha-synuclein, the fluorescence lifetime of ThT increased from 0.5 ns to 1-2 ns, as quenching by twisted intramolecular charge transfer was prevented. The results are shown in **Figure 5**.

Example 14 – Detection of alpha-synuclein oligomers with different dyes

In this example, the density of alpha-synuclein oligomers which could be detected with different dyes at a given alpha-synuclein concentration was determined. The dyes tested were ThT, diThT-PEG2, and DCVJ.

Alpha-synuclein was aggregated for 20 h at 37°C and agitation at a concentration of 350 μM as described in Chen *et al.* (2015) . All dye stock solutions were prepared in DMSO (SIGMA-ALDRICH) at a concentration of about 10 M and diluted into pre-filtered PBS (0.02 μm filter, WHATMAN) to a final concentration of ~100 μM. The final concentration of the dye solutions was determined from the corresponding absorbance for ThT at 412 nm with an extinction coefficient of 36,000 M^{-1} cm^{-1}; for DiThT-PEG2 at 410 nm with an extinction coefficient of 45,800 M^{-1} cm^{-1}; and for DCVJ at 460 nm with an extinction coefficient of 46,000 M^{-1} cm^{-1}.

Borosilicate glass coverslips (VWR INTERNATIONAL) were cleaned in an Argon plasma for 1 hour and prepared for coating with a 20 mm x 20 mm Frame-Seal slide chamber (BIO-RAD). The coverslips were coated with Poly-(L)-Lysine (PLL) (SIGMA-ALDRICH) for at least one hour. The PLL-coated surfaces were washed three times with PBS before the sample was applied. Alpha-synuclein was used at a concentration of 70 nM in PBS with varying concentrations of the according dyes.

Each sample was left on the coverslip for 10 min prior to imaging to ensure fixation of the species on the surface. The samples were imaged using a home-built total internal reflection fluorescence (TIRF) microscope as described in Example 5, limiting the detectable fluorescence signal to within 200 nm from the coverslip. For each dataset 3 x 3 image-grids were measured from three different areas of the coverslip with set grid distances to prevent user bias. Images were recorded at 50 ms exposure and 100 frames each field of view in the blue channel (ThT emission). Data analysis was done using custom written procedures using the GDSC SMLM plugin (SUSSEX UNIVERSITY) for ImageJ (http://imagej.nih./goc/ij) (Rasband, 1997-2014). In short, the image stacks were averaged over the full set and the background is removed and the 2D Gaussian point-spread-functions (PSF) were fitted using PeakFit. Signal to background threshold was set to 0.5 and spots were separated into 'oligomers' and 'fibrils' via an ellipticity threshold of 1.5. The number of total events was then divided by the image area to give the density of spots per μm^2. The results are shown in **Figure 6B**. Figure 6B demonstrates that di-ThT had a higher detection sensitivity than ThT. Use of DCVJ resulted in the "detection" of very high densities of oligomers at high dye concentrations. However, this was found to be the result of a very high false positive rate for this dye.

Example 15 – Detection of puncta in peripheral blood plasma

Having shown that β-sheet containing oligomers can be detected in CSF, the inventors tested whether the methodology was also suitable for detecting β-sheet containing oligomers in human peripheral blood plasma. The presence of alpha-synuclein in blood plasma, for example, has previously been reported (El-Agnaf et al., 2006). The method employed was essentially as described in Example 5, with the following differences. Peripheral blood plasma from a PD patient was diluted 10 x into PBS buffer before being placed onto a plasma cleaned poly-l-lysine coated coverslip. After 15 minutes incubation, the sample was washed 3x with clean PBS buffer. Finally ThT was added to produce a final concentration of 1μM in the sample. The sample was then imaged using TIRF microscopy and analysed using the inventors' bespoke image counting macro to determine the number of fluorescent puncta detected. These are highlighted using white boxes in **Figure 7**). The detection of fluorescent puncta in peripheral blood plasma is consistent with the detection of β-sheet containing amyloidogenic oligomers, as in the case of the CSF experiments reported in Example 11 above.

These data suggest that even though the autofluorescence background in peripheral blood plasma is much higher (~4.5 fold) than in CSF, detection of β-sheet containing oligomers in other biofluids such as peripheral blood plasma using the method developed by the present inventors is viable. The detection method developed by the inventors is therefore not limited to CSF samples.

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All documents mentioned in this specification are incorporated herein by reference in their entirety.

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Claims

1. A method of assessing whether an individual has a neurodegenerative disorder, the method comprising:

providing a cerebrospinal fluid (CSF) or peripheral blood plasma sample obtained from the individual,

introducing the sample to a fluorescent dye,

measuring a change in one or more photophysical properties of the fluorescent dye upon binding of the fluorescent dye to individual β-sheet containing amyloidogenic oligomers in the sample, and

determining the number and/or size of β-sheet containing amyloidogenic oligomers in the sample from the measured change,

wherein the number and/or size of β-sheet containing amyloidogenic oligomers in the sample indicates whether the individual has a neurodegenerative disorder.

2. A method according to claim 1, wherein a higher number of β-sheet containing amyloidogenic oligomers in the sample, compared with the number of β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma sample obtained from a healthy individual, indicates that the individual has a neurodegenerative disorder.

3. A method according to claim 1 or 2, wherein larger β-sheet containing amyloidogenic oligomers in the sample, compared with the size of β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma sample obtained from a healthy individual, indicates that the individual has a neurodegenerative disorder.

4. A method according to any one of claims 1 to 3, wherein the β-sheet containing amyloidogenic oligomers appear as diffraction limited features when imaged using a microscope set up that has a diffraction limit of 300nm or less.

5. A method according to any one of claims 1 to 4, wherein the β-sheet containing amyloidogenic oligomers have a diameter of 300nm or less.

6. A method according to any one of claims 4 to 5, wherein the β-sheet containing amyloidogenic oligomers have a diameter of at least 10nm.

7. A method according to any one of claims 1 to 6, wherein the β-sheet containing amyloidogenic oligomers consist of up to 200 monomers.

8. A method according to any one of claims 1 to 7, wherein the β-sheet containing amyloidogenic oligomers comprise, or consist of:

alpha-synuclein, and/or amyloid-beta, and/or tau; or

prion protein (PrP^{Sc}).

9. A method according to any one of claims 1 to 8, wherein the photophysical property is fluorescence intensity, fluorescence lifetime, Stokes shift, and/or polarization.

10. A method according to any one of claims 1 to 9, wherein the photophysical property is fluorescence intensity and/or fluorescence lifetime.

11. A method according to any one of claims 1 to 10, wherein measuring the change in the one or more photophysical properties is performed using a microscope set up for single-molecule microscopy.

12. A method according to claim 11, wherein the single-molecule microscopy is single-molecule fluorescence microscopy.

13. A method according to any one of claims 11 to 12, wherein the microscope is a confocal microscope, or a total internal fluorescence (TIRF) microscope.

14. A method according to any one of claims 11 to 13, wherein the lens of the microscope has a numerical aperture of 1.3 or higher.

15. A method according to any one of claims 11 to 14, wherein the microscope comprises a light source, and the illumination intensity of the light source is 50 W/cm^2 or more.

16. A method according to claim 15, wherein the light source is a laser.

17. A method according to any one of claims 1 to 16, wherein the fluorescent dye exhibits a Stokes shift of at least 20 nm.

18. A method according to any one of claims 11 to 17, wherein the microscope comprises a single-photon detector.

19. A method according to any one of claims 11 to 18, wherein the microscope is a TIRF microscope, and wherein the sample is diluted to achieve a separation of β-sheet containing amyloidogenic oligomers of at least 1 μm on the imaging surface of the microscope.

20. A method according to any one of claims 11 to 18, wherein the microscope is a confocal microscope and wherein the sample is diluted such that there is a low probability that more than one β-sheet containing amyloidogenic oligomer is present in the probe volume at any one time.

21. A method according to any one of claims 1 to 20, wherein the fluorescent dye exhibits at least a 1,000 fold increase in fluorescence quantum yield upon binding to a β-sheet containing structure.

22. A method according to any one of claims 1 to 21, wherein the fluorescent dye is selected from the group consisting of: dimeric Thioflavin T, Thioflavin T, Thioflavin S, Thioflavin-glycol, Pittsburgh compound B (PiB), 9-(2,2-Dicyanovinyl)julolidine (DCVJ), and analogues thereof.

23. A method according to claim 22, wherein the fluorescent dye is Thioflavin T, dimeric Thioflavin T, or DCVJ.

24. A method according to claim 23, wherein the fluorescent dye is Thioflavin T or dimeric Thioflavin T.

25. A method according to claim 24, wherein the fluorescent dye is Thioflavin T.

26. A method according to any one of claims 1 to 25, wherein the individual has been determined to be at risk of developing a neurodegenerative disorder.

27. A method of delaying or preventing the onset of clinical symptoms associated with a neurodegenerative disorder, the method comprising:

(i) assessing whether or not an individual has a neurodegenerative disorder using a

method according to any one of claims 1 to 26; and

(ii) administering to an individual assessed as having a neurodegenerative disorder in (i) a therapeutically effective amount of an agent capable of delaying or preventing the onset of clinical symptoms associated with said neurodegenerative disorder.

28. A method according to claim 27, wherein the method comprises administering to an individual assessed as having a neurodegenerative disorder in (i) a therapeutically effective amount of an agent which:

(a) directly or indirectly inhibits the formation of β-sheet containing amyloidogenic oligomers;

(b) an agent that directly or indirectly induces the degradation of β-sheet containing amyloidogenic oligomers; or

(c) binds to β-sheet containing amyloidogenic oligomers.

29. An *in vitro* method of identifying an agent capable of directly or indirectly inhibiting the formation of β-sheet containing amyloidogenic oligomers, the method comprising:

(i) providing a solution comprising monomers capable of forming β-sheet containing amyloidogenic oligomers;

(ii) incubating said solution under conditions suitable for the formation of β-sheet containing amyloidogenic oligomers in the presence and absence of said agent;

(iii) introducing the solution to a fluorescent dye;

(iv) measuring a change in one or more photophysical properties of the fluorescent dye upon binding of the fluorescent dye to individual β-sheet containing amyloidogenic oligomers in the solution, and determining the number and/or size of β-sheet containing amyloidogenic oligomers in the solution from the measured change; and

(v) comparing the number and/or size of β-sheet containing amyloidogenic oligomers formed in the presence of the agent with the number and/or size of β-sheet containing amyloidogenic oligomers formed in the absence of said agent;

wherein a lower number of β-sheet containing amyloidogenic oligomers formed in the presence of the agent than in the absence of said agent indicates that the agent is capable of directly or indirectly inhibiting the formation of β-sheet containing amyloidogenic oligomers; and/or

wherein smaller β-sheet containing amyloidogenic oligomers formed in the presence of the agent than in the absence of said agent indicates that the agent is capable of directly or indirectly inhibiting the formation of β-sheet containing amyloidogenic oligomers.

30. A method of identifying an agent capable of directly or indirectly inhibiting the formation of β-sheet containing amyloidogenic oligomers, the method comprising:

(i) providing a CSF or peripheral blood plasma sample obtained from a non-human animal that is a model of a neurodegenerative disorder, wherein the animal has been treated with the agent;

(ii) introducing the sample to a fluorescent dye;

(iii) measuring a change in one or more photophysical properties of the fluorescent dye upon binding of the fluorescent dye to individual β-sheet containing amyloidogenic oligomers in the sample, and determining the number and/or size of β-sheet containing amyloidogenic oligomers in the sample from the measured change;

wherein the number and/or size of β-sheet containing amyloidogenic oligomers in the sample indicates whether the agent is capable of directly or indirectly inhibiting the formation of β-sheet containing amyloidogenic oligomers.

31. A method according to claim 30, wherein the number and/or size of β-sheet containing amyloidogenic oligomers in the sample is compared with the number and/or size of β-sheet containing amyloidogenic oligomers in a CSF or peripheral blood plasma control sample obtained from a second non-human animal that is a model of the neurodegenerative disorder and which has not been treated with the agent,

wherein a lower number of β-sheet containing amyloidogenic oligomers in the sample obtained from the animal treated with the agent than in the control sample indicates that the agent is capable of directly or indirectly inhibiting the formation of β-sheet containing amyloidogenic oligomers; and/or

wherein smaller β-sheet containing amyloidogenic oligomers in the sample obtained from the animal treated with the agent than in the control sample indicates that the agent is capable of directly or indirectly inhibiting the formation of β-sheet containing amyloidogenic oligomers.

32. A method according to claim 30 to 31, wherein the animal is a rodent.

33. A method according to claim 32, wherein the animal is a rat or mouse.

34. A method according to any one of claims 1 to 33, wherein the neurodegenerative disorder is characterised by protein aggregation.

35. A method according to any one of claims 1 to 34, wherein the neurodegenerative disorder is Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, or a transmissible spongiform encephalopathy.

36. A method according to claim 35, wherein the neurodegenerative disorder is Parkinson's disease.

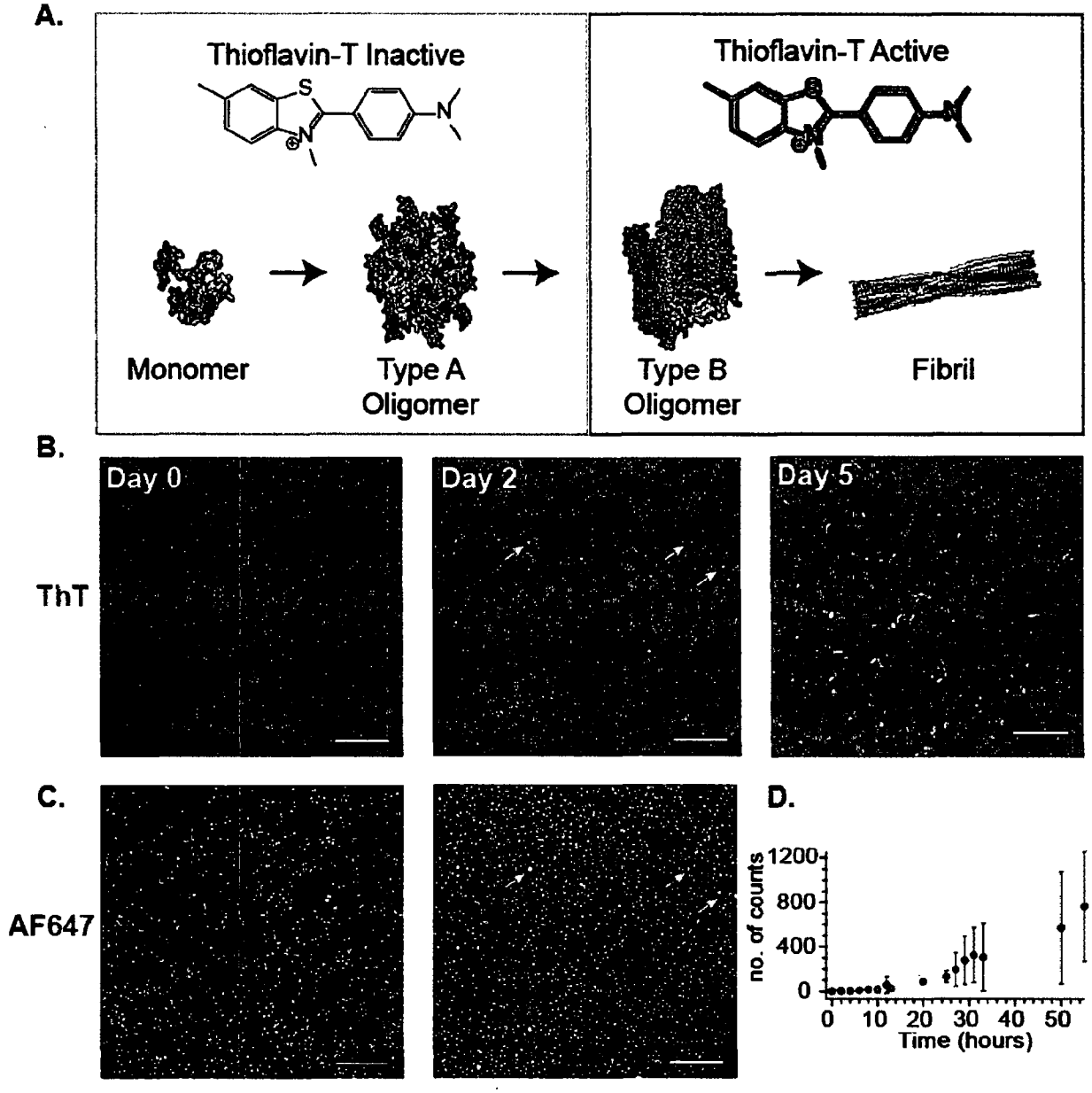

A.
Thioflavin-T Inactive
Thioflavin-T Active
Monomer
Type A Oligomer
Type B Oligomer
Fibril
B.
ThT
Day 0
Day 2
Day 5
C.
AF647
D.
no. of counts
1200
800
400
0
0 10 20 30 40 50
Time (hours)

Figure 1

E.

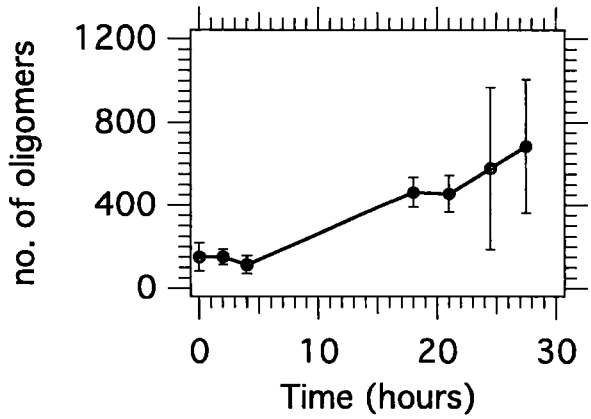


Figure 1 continued

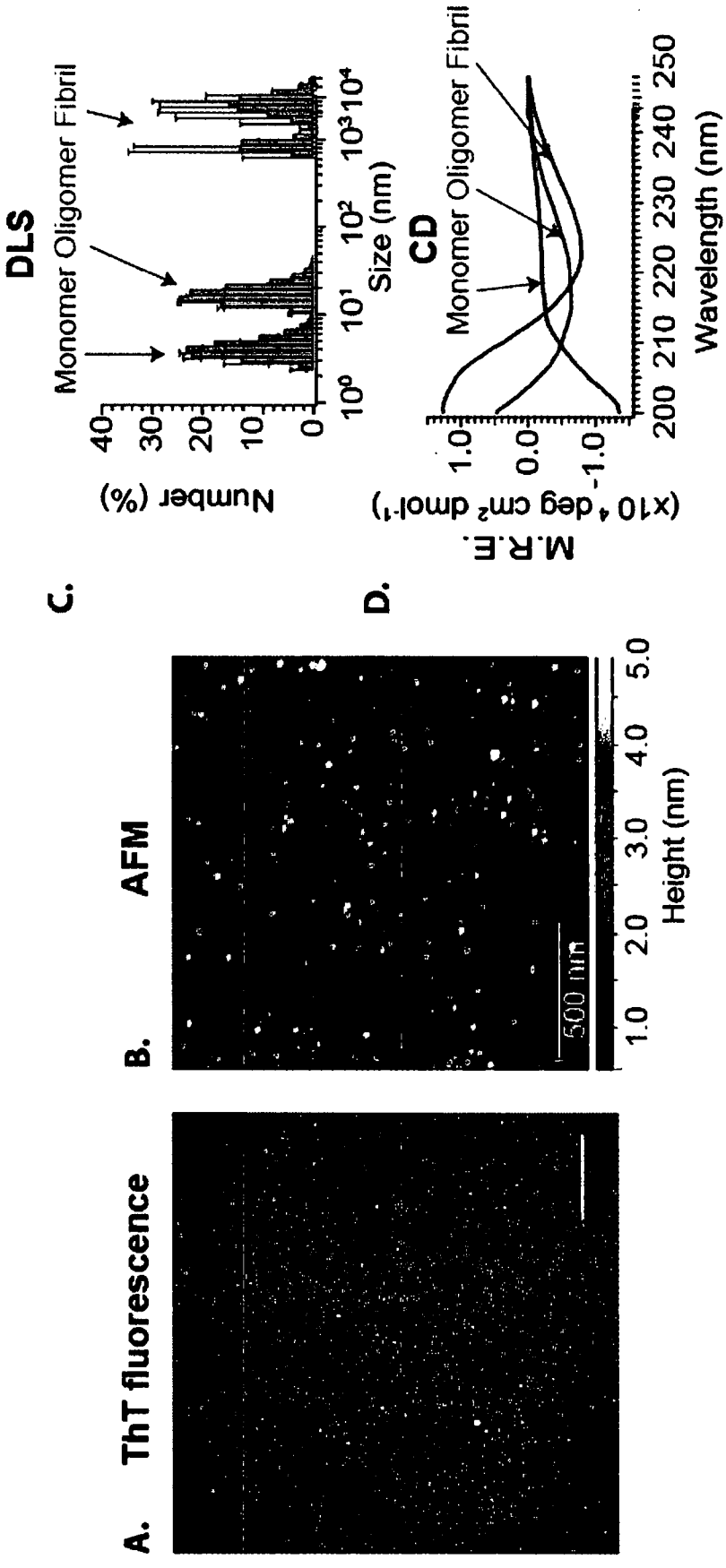

A.
ThT fluorescence
B.
AFM
500 nm
1.0
2.0
3.0
4.0
5.0
Height (nm)
C.
DLS
Monomer
Oligomer
Fibril
Number (%)
40
30
20
10
0
10^0
10^1
10^2
10^3
10^4
Size (nm)
D.
CD
Monomer
Oligomer
Fibril
M.R.E.
(x10^4 deg cm^2 dmol^{-1})
1.0
0.0
-1.0
200 210 220 230 240 250
Wavelength (nm)

Figure 2

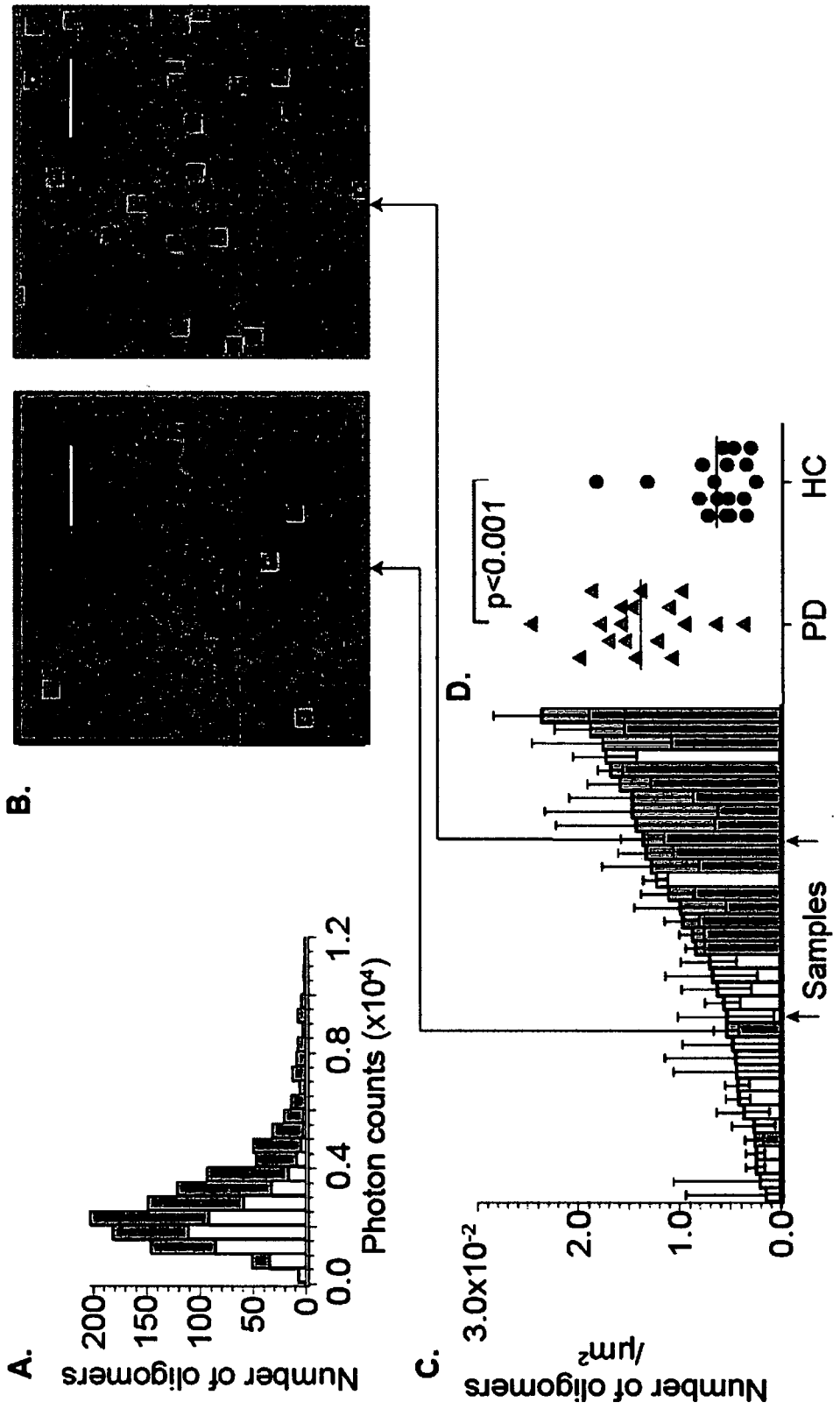

A.
Number of oligomers
200
150
100
50
0
0.0
0.4
0.8
1.2
Photon counts (x10⁴)
B.
C.
Number of oligomers /μm²
3.0x10⁻²
2.0
1.0
0.0
Samples
D.
p<0.001
PD
HC

Figure 3

E.

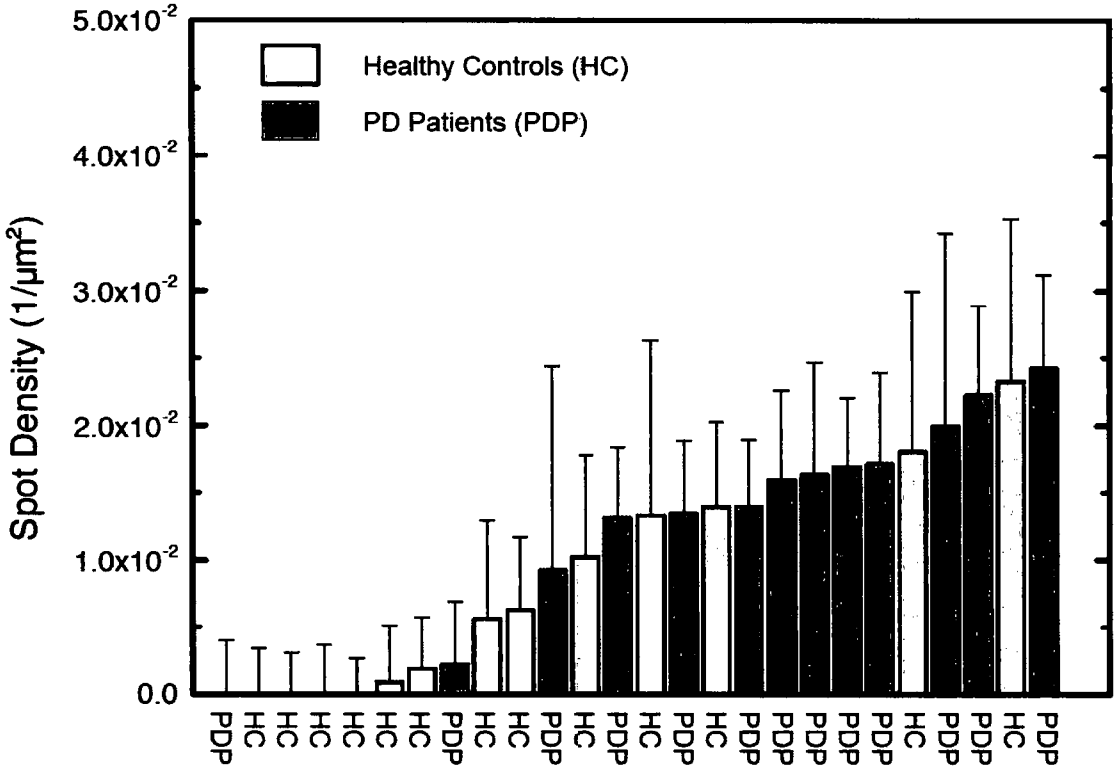


Figure 3 continued

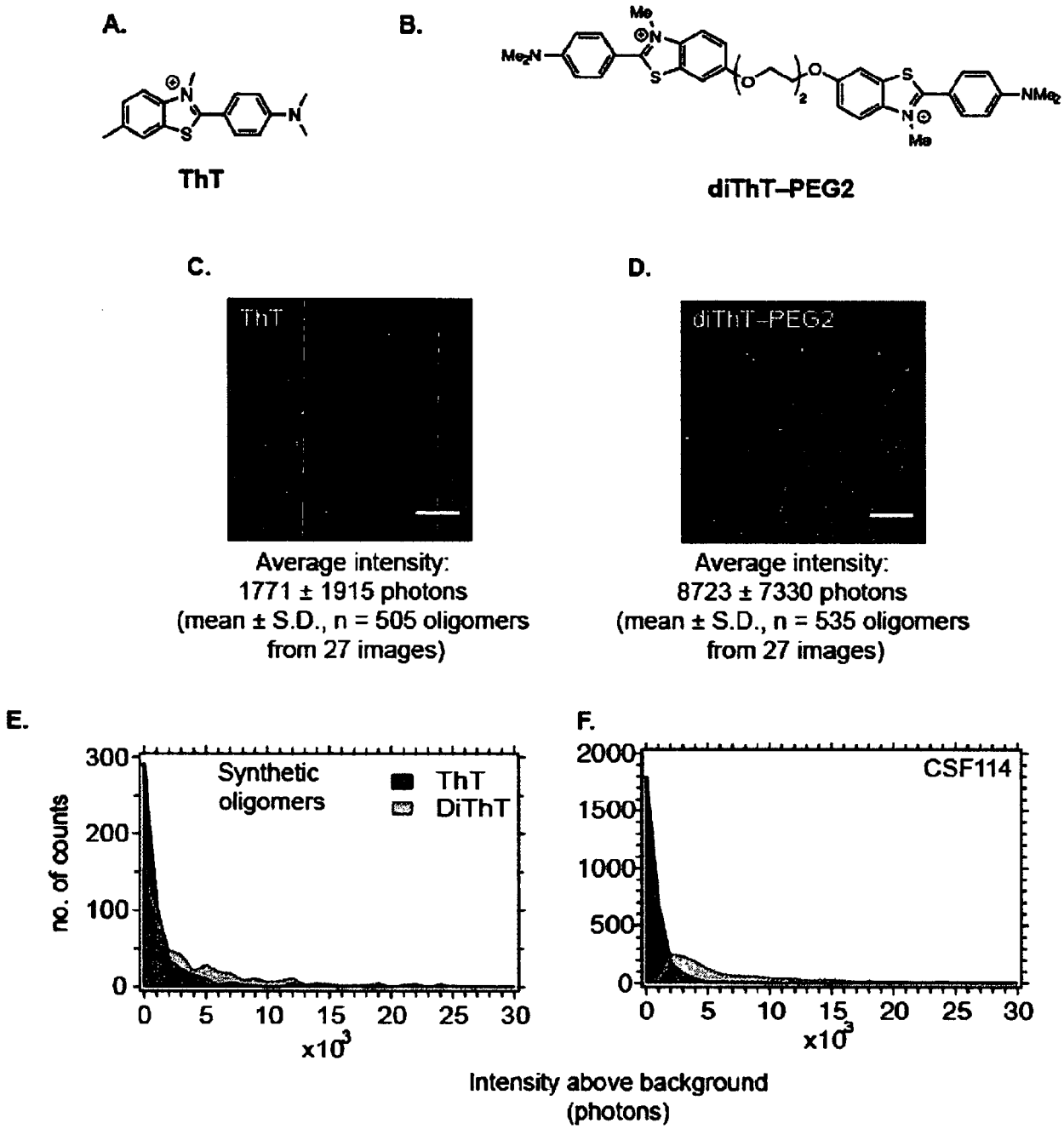

A.
ThT
B.
diThT-PEG2
C.
ThT
Average intensity:
1771 ± 1915 photons
(mean ± S.D., n = 505 oligomers
from 27 images)
D.
diThT-PEG2
Average intensity:
8723 ± 7330 photons
(mean ± S.D., n = 535 oligomers
from 27 images)
E.
Synthetic oligomers
ThT
DiThT
no. of counts
x10^3
F.
CSF114
x10^3
Intensity above background
(photons)

Figure 4

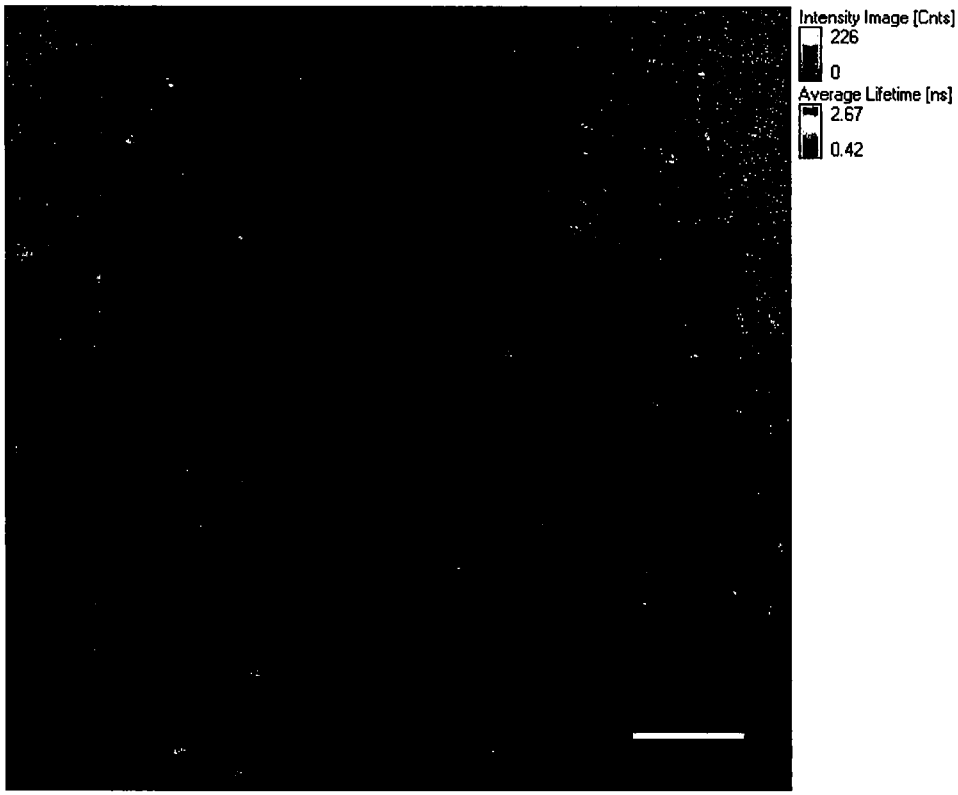
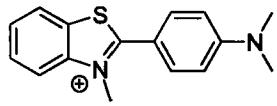


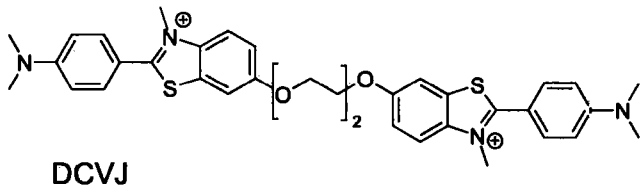
Figure 5

A.

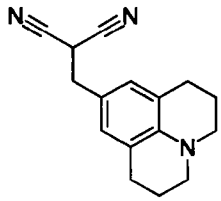
Thioflavin T



DiThT-PEG2



DCVJ



B.

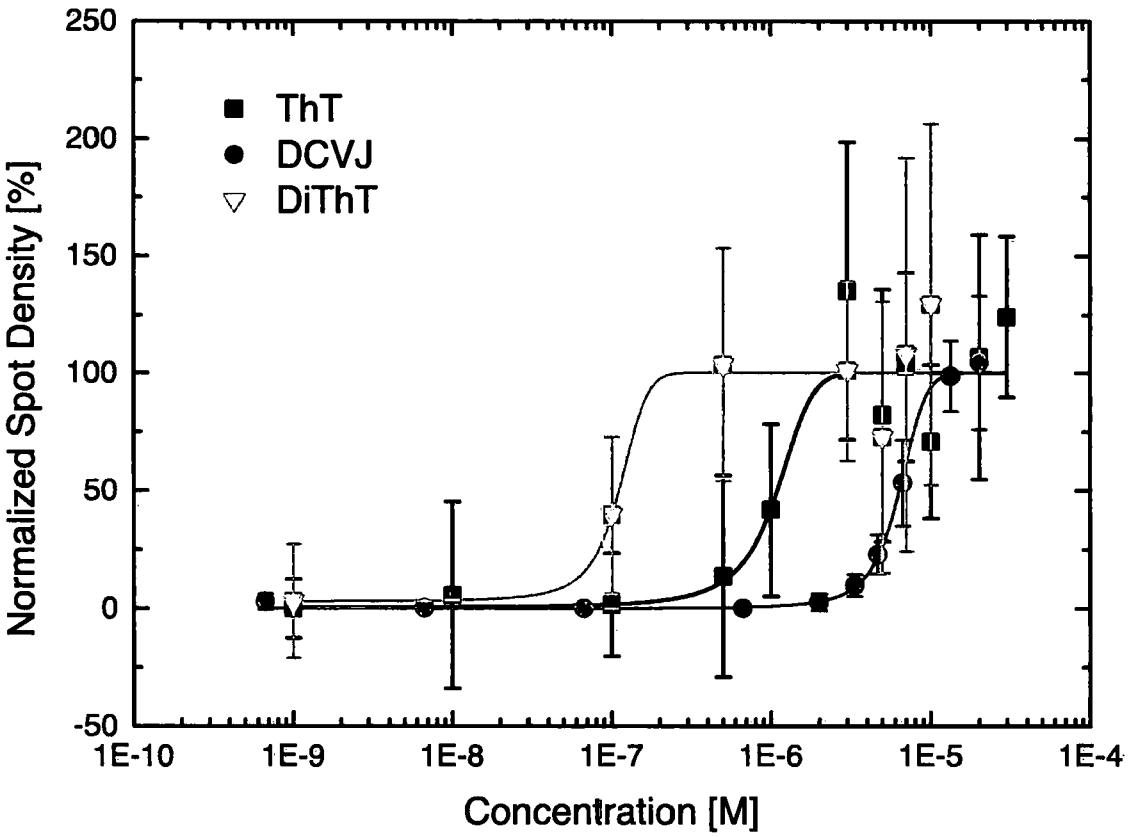


Figure 6



Figure 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/051899

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, MEDLINE, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2005/057166 A2 (ARETE ASSOCIATES [US]; ORSER CINDY [US]; GROSSET ANNE [US]; DAVIDSON E) 23 June 2005 (2005-06-23) claims 1-34	1-36
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
11 September 2015	23/09/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Moreno de Vega, C

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	BENSENY-CASES ET AL: "Conversion of non-fibrillar beta-sheet oligomers into amyloid fibrils in Alzheimer's disease amyloid peptide aggregation", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US, vol. 361, no. 4, 10 September 2007 (2007-09-10), pages 916-921, XP022240278, ISSN: 0006-291X, DOI: 10.1016/J.BBRC.2007.07.082 abstract page 917, left-hand column, paragraph 3 - page 919	1-36

Y	ONO ET AL: "Cerebrospinal fluid of Alzheimer patients promotes beta-amyloid fibril formation in vitro", NEUROBIOLOGY OF DISEASE, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, GB, vol. 20, no. 2, 1 November 2005 (2005-11-01), pages 233-240, XP005118658, ISSN: 0969-9961, DOI: 10.1016/J.NBD.2005.03.005 the whole document	1-36

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Y	the whole document	1-36

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ONO ET AL: "Cerebrospinal fluid of Alzheimer's disease and dementia with Lewy bodies patients enhances alpha-synuclein fibril formation in vitro", EXPERIMENTAL NEUROLOGY, ACADEMIC PRESS, NEW YORK, NY, US, vol. 203, no. 2, 27 January 2007 (2007-01-27), pages 579-583, XP005737599, ISSN: 0014-4886, DOI: 10.1016/J.EXPNEUROL.2006.08.012 the whole document	1-36
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Y	abstract page 2756, right-hand column, paragraph 1	1-36
A	WO 2011/033252 A1 (UNIV UNITED ARAB EMIRATES [AE]; EL-AGNAF OMAR [AE]) 24 March 2011 (2011-03-24) claims 1-20	1-36
X	LIU JINGGONG ET AL: "Synthesis and characterization of 1H-phenanthro[9,10-d]imidazole derivatives as multifunctional agents for treatment of Alzheimer's disease", BIOCHIMICA ET BIOPHYSICA ACTA (BBA) - GENERAL SUBJECTS, ELSEVIER, AMSTERDAM, NL, vol. 1840, no. 9, 10 May 2014 (2014-05-10) , pages 2886-2903, XP029015592, ISSN: 0304-4165, DOI: 10.1016/J.BBAGEN.2014.05.005	29,34,35
Y	page 2893, right-hand column, paragraph 2 - page 2894, left-hand column, paragraph 1	1-36

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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