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REVIEW

Detection of misfolded protein aggregates from a clinical perspective

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ABSTRACT

Neurodegenerative Protein Misfolding Diseases (PMDs), such as Alzheimer's (AD), Parkinson's (PD) and prion diseases, are generally difficult to diagnose before irreversible damage to the central nervous system damage has occurred. Detection of the misfolded proteins that ultimately lead to these conditions offers a means for providing early detection and diagnosis of this class of disease. In this review, we discuss recent developments surrounding protein misfolding diseases with emphasis on the cytotoxic oligomers implicated in their aetiology. We also discuss the relationship of misfolded proteins with biological membranes. Finally, we discuss how far techniques for providing early diagnoses for PMDs have advanced and describe promising clinical approaches. We conclude that antibodies with specificity towards oligomeric species of AD and PD and lectins with specificity for particular glycosylation, show promise. However, it is not clear which approach may yield a reliable clinical test first.

Relevance for patients: Individuals suffering from protein misfolding diseases will likely benefit form earlier, less- or even non-invasive diagnosis techniques. The current state and possible future directions for these are subject of this review.

1. Introduction

The oligomerization and then fibrillation of misfolded proteins is a common feature of a large group of diseases referred to as protein misfolding diseases (PMDs). A subset of these diseases is known as neurodegenerative because they cause irreversible damage to the central nervous system (CNS). Several different proteins may precipitate the clinical symptoms of these conditions, several parts of the CNS may be damaged and the mechanism of the condition varies (Table 1). The best known examples of neurodegenerative PMDs include Parkinson's [1,2] and Alzheimer's [2-4] diseases (PD and AD, respectively). Prionic diseases are less common and less predictable than the others but their immediate clinical impact can be more dramatic [5,6]. There are also a number of hereditary conditions also counted among the neurodegenerative PMDs, the most well- known of these being Huntington's Disease (HD) [7]. While the exact nature and relevance of protein misfolding is sometimes debated, for instance the relevance and nature of Huntingtin (Htt) aggregation in HD [8,9], there is agreement that misfolded, mis-aggregated or wrongly processed proteins are the unifying feature of these conditions

List of abbreviations
Alzheimer's Disease, AD; Amyloid Precursor Protein, APP; Beta-Amyloid, Aβ; Bovine Spongiform Encephalopathy, BSE; Creutzfeldt-Jakob Disease, CJD; Central Nervous System, CNS; Immunofluorescence, IF; Immunoprecipitation, IP; Enzyme Linked Immunosorbent Assay, ELISA; Huntingtin, Htt; Huntington's Disease, HD; Immunohistochemistry, IHC; Luminescent Conjugated Oligothiophenes, LCOs; Parkinson's Disease, PD; Prion Protein Cellular, PrPC; Prion Protein Scrapie-associated, PrPSc; Protein Misfolding Disease, PMD; α-Synuclein, α-Syn; Western Blot, WB.

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Table 1. Neurodegenerative protein misfolding diseases

Protein Misfolding Disease	Aggregating protein(s)	Aetiology	Clinical Manifestation	Pathogenic mechanism
AD	Aβ, Tau [128]	Acquired; age, gene variants increase risk, see also familial forms	Dementia, language difficulties, executive dysfunction, depression, hallucinations, delusions, agitation, apathy, disinhibition [128]	Depositions of Aβ plaques and Tau tangles observed. Selective loss of cholinergic neurones, loss of synapses and neurones in the cerebral cortex, atrophy of frontal cortex cingulate gyrus, temporal lobe and parietal lobe [129]
Cerebral amyloid angiopathy	Aβ, BRI2, Cystatin C, gelsolin, PrPSc, Transthyrin [130]	Acquired; age, familial factors and familial subtypes types identified	Cerebral haemorrhage, ischemic lesions, progressive dementia [130]	Progressive deposition of amyloid protein in cerebral blood vessel walls leading to degenerative vascular changes [130]
PD	α-Syn, Tau [131,132]	Acquired; head trauma, specific gene variants known to increase risk	REM sleep behaviour disorder, Excessive daytime sleepiness, hyposmia, depression, bradykinesia, rigidity, tremors, mild cognitive impairment, dyskinesia, dysphagia, postural instability, freezing of gait, orthostatic hypotension [131]	Manifestation of Lewy bodies enriched in α-Syn. Loss of dopaminergic neurons in the substantia nigra, Neuroinflammation with reactive gliosis and microgliosis [131]
Frontotemporal lobar degeneration	Tau, TDP-43, FUS, p62, ubiquitin [133]	Major genetic contributions [134]	Personality changes, behavioural disinhibition, apathy, progressive aphasia [53]	Neuronal loss, gliosis, microvacular changes of frontal lobes, anterior temporal lobes, anterior cingulate cortex and insular cortex [133]
Huntington's disease	Htt [135]	Congenital, monogenic	Mild psychotic and behavioural symptoms, progressive chorea, rigidity, dementia, dystonia, bradykinesia [135]	Gross striatal atrophy, neuronal loss in neocortex, cerebellum, hippocampus, substantia nigra, and brainstem nuclei [135]
Familial British dementia, and Familial Danish dementia	BRI2 [136]	Congenital, monogenic	Progressive cognitive impairment, spastic tetraparesis, cerebellar ataxa [137]	Amyloid angiopathy and neurofibrillary tangles (NFTs) in the hippocampus [136]
CADASIL, Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy	NOTCH3 [138]	Congenital, monogenic [139]	Mood disturbances, apathy, subcortical ischemic events, migraine with aura, cognitive impairment [138]	Degeneration of smooth muscle cells in blood vessels [138]
Alexander disease	GFAP [140]	Sporadic; gene variants increase risk	Macrocephaly, frontal leukodystrophy, palatal tremors, dysphagia, cognitive delays, seizures [140]	Demyelination, Rosenthal fibres in astrocytes [141]
Familial encephalopathy with neuroserpin inclusion bodies	Neuroserpin [142]	Congenital, monogenic [143]	Dementia, epileptic, seizures, progressive myoclonus, dysarthria [142]	Poorly understood, encephalopathy with neuroserpin inclusion bodies [142]
Kuru	PrP [144]	Acquired; transmitted	Cerebellar ataxia, choreifrom, athetoid movements, nystagmus, dysphasia [144]	Spongiform change, neuronal loss, astrocytic microgliosis, kuru plaques [144]
Creutzfeldt-Jakob disease	PrP [145]	Acquired; transmitted	Dementia, myoclonus, visual or cerebellar disturbance, akinetic mutism, pyramidal or extrapyramidal signs [146]	Spongiform change, neuronal loss, gliosis [145]
Gerstmann-Straussler-Scheinker syndrome	PrP [144]	Major genetic contributions [147]	Cerebellar ataxia, gait abnormalities, dementia, dysarthria, ocular dysmetria, myoclonus, spastic paraparesis, parkinsonism, hyporeflexia or areflexia in lower extremities [144]	Amyloid plaques, severe to absent spongiform changes, neuronal loss, astrocyte microgliosis, variable neurofibrillary tangles [144]
Fatal familial insomnia	PrP [144]	Congenital, monogenic [148]	Insomnia, myoclonus, ataxia, dysarthria, dysphagia, pyramidal signs, autonomic hyperactivation [144]	Neuronal loss, astrogliosis, hypometabolism in the thalamus and cingulate cortex [144]
Progressive supranuclear palsy	Tau [149]	Acquired; head trauma [150]	Progressive axial rigidity, vertical gaze palsy, dysarthria, dysphagia [149]	Neuronal loss, gliosis, neurofibrillary tangles affecting brainstem, basal ganglia, diencephalon [149]
Chronic traumatic encephalopathy	Tau, TDP-43 [151]	Acquired; head trauma [152]	Learning and memory impairment, anterograde amnesia, executive dysfunction, depression, apathy, irritability, suicidality, loss of impulse control, dementia, PD, dysarthria [151]	Atrophy of frontal and temporal cortices and medial temporal lobe, atrophy of the thalamus, hypothalamus and mammillary bodies. Thinning of the corpus callosum, pallor of the substantia nigra and locus coeruleus, cavum septum pellucidum [151]
Lytico-Bodig disease	Tau [153]	Acquired	Global dementia, progressive aphasia, gaze palsy, parkinsonism, progressive supranuclear palsy [153]	Poorly understood, neurofibrillary tangles are found in the brain [153]

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Protein Misfolding Disease	Aggregating protein(s)	Aetiology	Clinical Manifestation	Pathogenic mechanism
Meningioangiomatosis	Tau [154]	Acquired	Epileptic seizures, haemorrhagic stroke, anginoma, status epilepticus, generalized tonic–clonic seizures [155]	Focal lesion of the leptomeninges and underlying cerebral cortex [155]
Neuronal Ceroid Lipofuscinosis	ATP synthase subunit c, saposin A, saposin D [156]	Congenital, monogenic, subtypes exists [157]	Hypotonia, myoclonic jerks, generalized epileptic seizures, developmental regression, optic atrophy, macular degeneration, spastic tetraplegia, blindness, severe and constant microcephaly, and pharmaco-resistant epileptic seizures, myoclonia, ataxia, extrapyramidal signs [156]	Cerebellar and cortical atrophy, loss of pyramidal neurons and Purkinje cells, reactive astrogliosis [156]
Argyrophilic grain disease	Tau [158]	Acquired; old age [158]	Cognitive decline, dementia, mood imbalance, personality changes, behavioural abnormalities [158]	Argyrophilic grains in trans entorhinal cortex, entorhinal cortex, hippocampus, presubiculum, temporal cortex, orbitofrontal cortex, insular cortex, and amygdala[158]

[10]. Full recovery has not yet been observed in any patient after damage to nerve tissue has begun. Clear and early diagnosis of these conditions is therefore essential for informing sufferers about their condition, managing the condition where this is possible and giving appropriate palliative care. Theoretically, early diagnosis might help guide choice of treatment in cases where effective options are available.

The desire for both prompt diagnosis and improved medical treatments has thus encouraged research into misfolded protein aggregates and their relative PMDs. Diligent research has identified and characterised many of the individual proteins involved in these conditions. For example, β-amyloid (Aβ) [11] and Tau in AD [12], α-Synuclein (α-Syn) in PD [13,14] and Htt in HD [7] are now largely accepted to have key roles in these diseases (For other protein involvement in given diseases, see Table 1). Despite this, tests for diagnostic compounds (biomarkers) are not in routine use for identifying any of the non-hereditary PMDs. For instance, when assessing patients for AD, clinicians have to rely on an imaging or visual data regarding symptoms and standardized tests that are sometimes combined with MRI [7]. Biopsies for detecting changes in the CNS are considered invasive procedures that are generally unsuitable for elderly patients, and thus they are usually only used for verification of the diagnosis post mortem [15]. Importantly, tissue damage precedes the formation of the characteristic insoluble fibrils that are detected in brains of AD sufferers. Such fibrils are not cytotoxic and their formation correlates only poorly with disease progression [16]. This suggests that other agents, such as oligomers of the same misfolded proteins as the fibrils, are responsible.

The common features of neurodegenerative PMDs, in which only one or two proteins appear to be defective, or at least the processing of which is defective, is an attractive target for translational research aiming to detect the condition in its early stages. One possible diagnostic tool is therefore a detection system for the activity of proteases that are also involved in the progression of the disease, such as caspase-8 activation in the case of PD [17] and β- and γ-secretase in AD [18,19]. One problem with this approach is that such processes do not have a unique association with the diseases in question. Another approach would be to focus on the individual misfolded proteins at an early stage, rather than the insoluble plaques and reduction in tissue volume associated with advanced stages. Sensitive tools are required for the early detection of conditions where the underlying biochemical changes may be small or difficult to resolve. For example, only minute quantities of transmissible misfolded prions are required to precipitate Creutzfeldt-Jakob Disease (CJD) in humans [6], bovine spongiform encephalopathy (BSE) and scrapie in sheep [20]. Nor is it necessarily straightforward to detect changes in protein folding, aggregation or processing in bodily fluids. Despite these challenges, methods for monitoring such changes in the relevant proteins of PMDs are of clinical interest. In this review, we explore recent advances in translational research focused on detecting misfolded proteins in the context of early pre-fibril misfolding. We discuss these advances from both a research and a clinical perspective. We conclude with a forward-looking view on possible research directions.

2. Protein misfolding, oligomerisation and toxicity

Proteins pass through a fundamental process called folding in order to obtain their functional structure [21-23]. Folding is usually spontaneous under physiological conditions, and occurs at rates that depend upon the protein's size [24]. This process takes a couple of hundred milliseconds for most proteins [25]. Early steps in protein folding include the clustering of hydrophobic amino acids and the expulsion of water, the subsequent compaction of the polypeptide chain and consolidation of secondary structure. Then, a re-ordering and fine-tuning of the structural elements takes place to afford the final tertiary structure. The folding process needs to make particular intra-fold contacts both in its early and late stages. All these steps are affected by the protein's environment. Factors that affect the outcome of a folding process and aggregation behaviour include solvent conditions, the presence or absence of cofactors and metal cations [26], chaperones or other dissolved factors, crowding from other proteins or macromolecular aggregates, spatial organization and post-translational modifica-

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tion (reviews [27,28]). The fact that folding is influenced by many factors is also reflected in the cell biology of the PMDs. The state of the prion protein associated with CJD is both translocated into the ER and glycosylated differently than its non-pathogenic counterparts [29,30]. Copper and zinc ions are implicated in both AD and PD, as there is evidence that they influence disease onset and progress in animal models and have been highlighted in clinical studies [31]. It is not entirely clear whether Cu^{2+} and Zn^{2+} are only involved in the misfolding of proteins or also in the aggregation of those proteins into fibrils [32].

Amyloid fibrils are widely recognized as a result of protein misfolding and have been observed both *in vitro* and *in vivo*. They are repetitive sheets in which monomers are joined by hydrogen bonds across β-strands. The long sheets are slightly twisted, with varying dimensions and crossover distances depending on the polypeptide involved. Cryo-electron microscopy has indicated that in Aβ the fibril is approximately 4 nm and 11 nm at the narrowest and widest points respectively and has a twist crossover distance that has a mean of about 100 nm. Comparative work on fibril morphology from Aβ and α-Syn indicates that they are similar, but with a degree of polymorphism [33-35]. A considerable range of proteins and protein fragments can form fibrils, suggesting that the barrier to formation of these states is more likely to rest with time and physico-chemical conditions than amino acid sequence.

Importantly, the extent of fibril formation does not appear to correlate with disease progression and naturally occurring mutants associated with the early onset of PMDs do not produce more fibrils [16]. For these reasons, attention has been given to the aggregates preceding fibril formation. It has been proposed recently that the toxic oligomers are, just like fibrils, a general phenomenon that forms relatively independently of protein sequence [36]. The oligomerisation of other proteins such as calcitonin [37], α-Syn [38], Syrian hamster prion protein [39], GAP-43 and BASP1 [40] is consistent with this. The oligomers have since been shown to display significant toxicity-related effects relative both to monomers, fibrils and proto-fibrils [41,42]. Furthermore, rates of their formation are better able to account for disease-promoting mutations [16,37,42,43]. It has also been suggested that their toxicity is linked to membrane damage through a pore-like action [44] and a range of pre-fibrillar oligomeric structures from a several proteins, including Aβ, Htt, prion proteins, and α-Syn, has since been investigated in this context [39,43,45,46].

3. Misfolded proteins and the lipid profile of the membrane

It is well established that membrane or peripheral proteins may affect membranes and *vice versa* (review [47]). The lipid-dependent, differential processing of APP to Aβ is one particularly relevant example of this [48]. Moreover, PMD proteins and notably their oligomeric states have considerable effects on membrane integrity. Furthermore, tissue deposits of amyloid fibrils contain lipids [49]. Imaging studies on pre-fibrillar oligomers reveal a range of structures, some of which may have a pore-like morphology (review [50]). These are referred to as amyloid pores or sometimes annular oligomers [51]. Whether such oligomers will go on to form mature amyloid fibrils exclusively is not clear, as there exists reports in which pore-like oligomers do not appear to undergo fibrillation [52]. Certain drugs can arrest fibril but not oligomer formation [53]. Even though the presence of non-fibrillar, pore-like oligomers correlates better with toxicity and disease-promoting mutants than fibrils, their properties, mechanism of action and what promotes or suppresses their formation remains poorly understood.

Two competing hypotheses that may explain the effect of oligomers on the membrane are being researched at the moment. The first suggests that oligomer toxicity is a direct result of pore formation. Examples of oligomers that may effect pore formation include those generated by islet amyloid polypeptide [54-57], poly-glutamine [58], transthyretin [59], prion protein fragment [60], Aβ [61], β2-microglobulin [62] and serum amyloid A [63]. Porosity was indicated by ionic flux across reconstituted membranes, which compromises cellular homeostasis and membrane potential [61]. The second hypothesis being tested at present is that the oligomers cause membrane thinning rather than leakage, through a distinct pore [42,64,65]. In this scenario, leakage through the membrane is independent of the pore-like aggregate morphologies and can take place through any area of the membrane that is sufficiently perturbed by these aggregates. Membrane thinning involves the increase of area per lipid and intercalation of polypeptides and water molecules between head groups in order to avoid energetically costly vacuums in the lateral lipid packing. This has the effect of lowering the dielectric barrier and allowing ion leakage through the membrane [10,66].

Experimental determination of leakage through a pore formation or through thinning of the membrane is not straightforward. Regardless of the particular mechanism, the oligomers convey toxicity by perturbing the integrity of the membrane. However, the membranes may in turn affect the oligomers, too. Aggregating, oligomeric peptides have been shown to have preferential binding to particular membrane components, in particular sphingolipids and cholesterol. Sphingolipids and cholesterol are found in patches termed the liquid ordered phase that are often referred to as lipid rafts [67], though controversy about this link exists [68]. Glycosphingolipids and gangliosides have affinity for the Aβ peptide in AD and α-Syn interacts with GM_1 and GM_3 gangliosides [69-74]. PrP has been associated with sphingolipid signalling platforms and bind to sphingomyelin, GalCer, GM_1 and GM_2 [70,75,76]. A strong interaction with sphingolipids may reflect the amount of amyloidogenic protein found in possible lipid raft areas of the extracellular leaflet of the plasma membrane [49]. Amyloidogenic proteins such as α-Syn also interact more strongly with anionic lipids phosphatidylglycerol and phosphatidylserine, found mainly in the cytoplasmic leaf [45,77-79]. Model systems comprising the anionic lipids cardiolipin, phosphatidic acid or phosphatidylglycerol leak more

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on contact with oligomers [45].

The role of cholesterol in membrane behaviour has been researched in some depth [80-88] but its role in oligomer formation and amyloidogenesis remains disputed and controversial [89]. Cholesterol has been shown to bind to Aβ proto-fibrils, but how these interactions influence oligomerization and later fibrillogenesis remains unclear [90-92]. There is evidence that cholesterol can have a stabilizing effect on membrane permeability as it reduces the leakage induced by α-Syn [46]. The role of cholesterol in the proteolysis of APP to give Aβ is better understood. Proteolysis of APP is inhibited by the group of cholesterol synthesis inhibitors known as statins [93,94]. In PD a depletion of cholesterol leads to a decreased level of α-Syn in membrane fractions in neuronal cell cultures and mouse brains [95]. Inhibition of cholesterol synthesis also reduces the levels of α-Syn in membranes, but the opposite applies to cholesterol supplementation in neuronal cells [96]. It has also been suggested that oxidised cholesterol accelerates aggregation of α-Syn [97].

This evidence may be at odds with the observation that cholesterol protects artificial membranes against oligomer-induced leakage [46] as it fails to provide a direct connection to the proposed toxicity mechanism. Polyunsaturated fatty acids may have an inhibitory role in oligomerization. The presence of docosahexaenoic acid (DHA) suppresses the toxicity of Aβ towards SH-SY5Y cells by interfering with its aggregation [98,99], and appears to have a neuro-protective role in murine models for AD [100]. However, DHA can also affect the progress of some cells through the cell cycle [101]. The notion that saturation levels of the fatty acid residues (FARs) of phospholipids in membranes play a role in modulating the rate of oligomerization agrees with measurements from model systems that indicate that saturated fatty acid residues lower the energetic barrier to aggregation [102].

Further work is required to understand the complexities of the relationship between membrane components and protein misfolding and oligomerization. It is possible that certain lipids or other membrane components may be used as diagnostic compounds for more reliable early-stage detection of neurodegenerative PMDs in combination with detection of the oligomeric proteins, should the links between lipid species and oligomerization prove robust.

4. Clinical detection of protein aggregates

There is no single rigorous assay for diagnosis of any PMD. The mounting evidence for the involvement of toxic oligomers in neurodegeneration confers an increasing importance on detection methods for basic and translational research, and in clinical practice. As a result, great research effort is being focused on developing clinical methods for detecting the main pathological unit of AD. At present, diagnosing AD includes a test of cognitive impairment (The Mini Mental State Exam or Folstein test), in some cases supplemented by CSF assays for phosphorylated tau and Aβ, MRI for brain volume and PET scans for Aβ plaques (or glucose metabolism) in the brain [103].

An overview of methods for clinical detection of protein aggregates is shown in Table 2. Generally, approaches for the identification of protein aggregates can be divided into three classes of method: (i) visualization of protein aggregates in biopsies, (ii) monitoring of marker peptide in bodily fluids, and (iii) visualization of protein aggregates *in vivo* using imaging techniques. Most of the methods discussed here concern Aβ peptide detection in AD, as this field has advanced the furthest. The majority of approaches rely on antibodies to confer specificity to the detection, whether it occurs in biopsies, bio-fluids or *in vivo*. A considerable number of different antibodies have been developed in the last two decades, many of which have at least some degree of specificity towards the proteins and aggregation-states involved in neurodegenerative PMDs. An overview of some of their properties is shown in Table 3a.

The visualization of amyloid plaques in samples from biopsies is a well-established means for qualitative detection of mature fibrils. There are several standard stains, such as Congo red and fluorescent thioflavins [104], as well as immunohistology stains based on antibodies [105]. There have been several recent advances in the development of fluorescent probes based on luminescent conjugated oligothiophenes (LCOs) which can be used for investigating the nature of these protein deposits [106,107]. LCOs are able to illuminate more protein deposit plaques than other fluorophores [108]. Moreover, the emission spectra of LCOs are dependent on the type of predominant peptide present. This makes it possible to distinguish e.g. AD-associated aggregates from other types of aggregates [109,110]. Another new and promising way of detecting of amyloid plaques is the discovery of photo-induced electron transfer probes that can be used to detect Aβ aggregates without the need of a washing step [111]. These recently-developed fluorescent probes represent a new opportunity in direct and sensitive identification of protein aggregates, especially in complex biological environments.

The second class of methods for the detection of proteins and their aggregates detects molecules in bio-fluid samples, avoiding the need for biopsies. These clinical methods rely on the detection of marker peptides in cerebrospinal fluid (CSF). For instance, detection of Aβ or tau protein in AD has been shown to have predictive power over which individuals will go on to develop the disease [112]. Detection of the relevant molecular species in CSF is relatively straightforward and a broad array of methods exists for its detection. It is possible to detect and quantify tau peptides in the lower ng/mL range using mass spectrometry on samples acquired directly from the CNS [12], although this not in routine clinical use yet.

A more common method is the use of enzyme-linked immuno-sorbent assays (ELISA) [113]. For example, antibodies 2G3 and 21F12 are used for the detection of C-terminal amino acids of Aβ peptides 1-40 and 1-42, respectively, in diagnosis of AD [114]. The same peptides can be detected by new electrochemical detection immuno-sensors. These biosensors are based on immobilization of antibodies on gold nanostructured

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Table 2. Clinical detection of protein aggregates

Proteopathy	Misfolding/oligomerizing protein	Current methods for clinical detection
AD	Aβ peptide	Decrease of marker peptide concentration in CSF, detected by ELISA, immuno-sensors [112,113,115]
		MRI with plaque selective magnetic nanoparticles – hollow manganese oxide nanoparticles coated with antibody or curcumin-conjugated magnetic nanoparticles [123]
		Fluorescent labelling of biotic samples with luminescent conjugated oligothiophenes [106,107,109-111]
		Late phase PET imaging of cerebral fibrillary Aβ peptide with 11C-Pittsburgh compound B as a PET ligand [159,160]
	Tau protein	Ratio of phosphorylated Tau in position 396 and 404 in CSF could discriminate AD from other dementia; Identification by ELISA [161]
		Identification of phosphorylated biomarkers pTau181, pTau199 and pTau231 in CSF by immunoassays [162-167]
Cerebral amyloid angiopathy	Aβ peptide	Early phase PET imaging of cerebral fibrillary β-amyloid with 11C-Pittsburgh compound B as a PET ligand [168-170]
PD	α-Syn	Detection of α-Syn aggregates in biotic samples by imunohistochemical or fluorescent staining [171-173]
		Multi-parametric fluorescent pyrene-labelling of biotic samples [174]
		Detection of α-Syn oligomers in human plasma or red cells by ELISA [175,176]
Huntington's Disease	Htt	Loss of brain volume observed by MRI with combination of genetic test – identification of abnormal CAG expansion in exon1 of htt gene [177-179]
Variant Creutzfeldt-Jakob disease	Prion protein PrP	Whole blood immunoassay [180-182]
Other prion diseases: Gerstmann-Sträussler-Scheinker disease, fatal familial insomnia, kuru, Creutzfeldt-Jakob Disease	Prion protein PrP	Conformation dependent immunoassays in biopsy samples[183,184]
		Analysis of 14-3-3 and PrPSc expression pattern in CSF [185]
		Detection of PrPSc in urine by immunoassay [186,187]

screen-printed electrodes with cyclic voltammetry detection [115], or with difference pulse voltammetry detection with immobilization on gelsolin coated electrodes that selectively binds Aβ peptides [116]. Another interesting approach combines ELISA and surface plasmon resonance to provide greatly enhanced detection, using gold nano-particles conjugated with antibodies [117]. The role of the nano-particles in this assay is to increase the change in refractive index response that each immobilized molecule produces. This provides detection limits as low as single molecules. This technique is also designed to handle precipitates as part of the detection assay, which may be an advantage when working with oligomerization states.

The last group of methods allow direct observation of amyloid plaques in vivo. This direct observation is appealing for clinical use, but is not yet practiced routinely. Methods like magnetic resonance imaging (MRI), positron emission tomography (PET) [118] and diffusion-tensor imaging [119,120] are being developed for direct diagnosis of amyloid plaques based on visual inspection of advanced imaging output. However, all of these methods are based only on qualitative approaches and rely on detecting visible changes in the CNS. There has been some work on quantification of amyloid loads based on PET image analysis but with very limited results [118,121]. More recent advances in MRI are based mainly on the use of particles that allow localization of particular plaques. For example it is possible to use curcumin-conjugated magnetic nano- particles [122] or hollow manganese oxide nano-particles conjugated with a particular antibody [123]. Both of these nanoparticle methods increase the specificity and sensitivity of the techniques towards the protein aggregates. However, these approaches are not in routine use and may not satisfy the need for diagnosis before irreparable damage to tissue has occurred.

5. Concluding remarks and future perspectives

The methods available for detecting proteins associated with neurodegenerative PMDs shows some promise for clinical use. However, many of these methods make no distinction between monomers or oligomers. Thus, the preparation of diagnostic tools that monitor the advancement of oligomerization at an early stage is still under development.

Antibodies are one of the most well established and still promising directions for developing diagnostic tools. Antibodies with ligand conformation sensitivity could be used to build one or more specific standardized clinical ELISA assays for detection of misfolded oligomers. The reliability of such immuno-based approaches is limited by the quality of the antibody involved. Notably, antibody-based detection of the misfolded oligomers, for instance Tau and Aβ, has advanced in recent years (see Table 3a), indicating that standard assays based on immunology may indeed be made sensitive to oligo-

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meric forms. Ensuring that there is a low limit of detection in a complex bio-fluid is also a concern, for ensuring early diagnosis.

Another approach could be the use of native mass spectrometry for detecting protein aggregates. It is a technique which, in contrast to other types of mass spectrometry, can detect non-covalent interactions between proteins [124]**.** This technique is also able to detect protein complexes across a wide range of molecular masses and handles heterogeneous samples well. All these features are attractive when aiming to detect oligomers in complicated samples such as bio-fluids. Native mass spectrometry has been used successfully to investigate the assembly of virus capsid, directly from crudely purified culture extract [125]. In principle, it is also possible to detect the oligomers discussed above. An overview of recent, promising use of mass spectroscopy in neurodegenerative PMDs can be found in Table 3c.

The detection of protein glycosylation may be a means for detecting prionic diseases at an early stage (See Table 3b for references). This approach relies upon detailed knowledge of the glycosylation chemistry involved. Although mass spectrometry may be helpful in identifying prion protein glycosylation species, the most promising tool at present are lectins. These are saccharide-binding proteins that can detect differences in glycosylation with some specificity and made a distinction between normal and disease-associated prionic protein successfully [126]. One limit to this approach is the ubiquity of glycosylation; it may not be clear which protein the sugars are actually attached to. For these reasons, false positive results

Table 3a. Oligomeric protein states detected by antibodies

Aggregating protein	Antibody	Specificity and epitope	Cross-reactions	Detection Methods and References
Aβ-peptide	4G8	Recognizes residue 18-23 in Aβ sequence, in its fibrils and fibrillary oligomers form	α-Syn, IAPP, Tau These cross-reactions are reported to be fibril-associated, not sequence dependent	WB, IHC, IP, ELISA [188-190]
	A11	Prefibrillar oligomers, not monomers or fibrils	Weakly detects annular oligomeric conformations from α-Syn and IAPP	WB, IHC [189,191]
	6E10	Amino acid 4-9 in Aβ sequence	Detects monomers, oligomers and fibrils, but does not cross-react with α-Syn or IAPP	WB, IHC, IP, ELISA, EM [189,192]
Tau	T22	Human Tau oligomers; conformationally specific epitope	Reported to have no significant cross-reaction with monomers or fibrils of Tau, or with α-Syn, IAPP, or Aβ in any form	WB, IHC, IP ELISA [193-195]
	TOMA	Human Tau oligomers; conformationally specific epitope	No cross-reaction with Tau monomers or fibrils, or with Aβ or α-Syn	WB ,IHC, ELISA [193]
α-Syn	Syn211	Amino acid 121-125 of human α-Syn sequence	Does not cross-react with mouse or rat subtypes. Does not cross-react with β-Syn or β-Syn.	WB, IHC, IP, IF [164]
	Syn-O2	Oligomers, weakly recognizes residue 127-140 of the α-Syn sequence	Does not detect monomers, but detects some fibril	WB, IP, IHC [196,197]
Huntingtin (Htt)	3B5H10	PolyQ in a compact β-strand configuration	Recognizes diseased-associated Htt from human and murine origin; no detectable binding to normal Htt	WB, IHC, IP, ELISA [8,198-200]
	MW1	PolyQ domain of Htt exon 1	Recognizes diseased-associated Htt from human and murine origin; no detectable binding to normal Htt	WB, IHC [200,201]
Prion protein PrPC and PrPSc	6D11	PrpC, human origin; epitope within residues 93-109	Also detects PrPSc. Cross-reacts with prion proteins from cervines, ovine, murine and cricetine	WB, IHC, ELISA, IF [202,203]
	G-12	Amino acid 217-232 human sequence	Murine, human	WB, IP, IF and ELISA [204]
	PRC5	Needs Ala in position 136	PrP from murine, cervines, bovine, ovine, equine, cricetine, mustelines, sciurine, primates	WB [205]
	D18	PrPC, conformationally specific epitope related to Helix 1, residues 130-160	Murine, human, recognises PrPC Only	WB [206-209]
	ICSM18	PrPC, conformationally specific epitope related to Helix 1, residues 130-160	Murine, human, recognises PrPC Only	WB [207-209]
	6H4	PrPSC conformationally specific epitope related to Helix 1, residues 130-160	Also recognises PrPC	WB [207-210]

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Table 3b. Identification of prion protein glycosylation states

Prion Protein Glycosylation state	Detection method	Sample type	References and notes
Preferential detection of aglycosyl and mono-glycosyl	Antibody PRC7; conformationally specific epitope. Residues at position 154, 166, 185 and 197 are involved.	Extract, WB	The epitope is glycosylation-dependent and residues 154 and 185 are involved [205]
Sialylated and O-glycosidically linked glycans	Lectin proteins affinity for specific glycosylations	Tissue, IHC	Antibodies for PrP (MAB1562 and AB5058) used to ensure that lectins actually detected prion proteins [126]
Glycoproteome of prion protein variants	708 proteins or protein variants assessed	Murine plasma samples	Combined MS-affinity chromatography based approach [211]

Table 3c. Identification of oligomeric states by mass spectrometry

Protein	Oligomeric state detected	Sample type	MS detection method sub-type	Reference
α-Syn	Differentiates between oligomers and monomers	Prepared from isolated protein	Hydrogen-Deuterium Exchange, ESI-MS	[212]
α-Syn	Monomers and oligomers	Prepared from isolated protein	ESI-ion mobility mass spectrometry	[213]
α-Syn	Differentiates between oligomers and monomers	Conditioned cell media, similar in complexity to Cerebrospinal Fluid	Combined MS-antibody based approach	[214]
APP, Prion Protein, DJ-1	Monomers and oligomers	Cerebrospinal Fluid	Tandem MS/MS	[215]
Prion Protein	Differentiates between PrPC and PrPSc	Samples prepared from brain homogenate	Quantitative LC–MS/MS	[216]

may be a significant problem unless the detection method can also identify the protein involved clearly. Fortunately, many well-stablished antibodies may help solve this problem (Table 3a), although extensive glycosylation may sometimes obscure the epitopes involved.

The problems inherent in the types of detection discussed here—low concentration of target protein, subtle differences between correctly and incorrectly folded and aggregated proteins, heterogeneous protein modifications and strong background signals when detecting in a complex biological environment—do not have obvious solutions. However, it seems likely that protein affinity-based techniques (antibodies, lectins) can successfully be combined with instrument-based detection methods, such as mass-spectrometry, fluorescence, and surface plasmon resonance to produce sensitive detection methods able to identify both the aggregation state and modification state of the protein in question. Moreover, there is reason to believe that early detection can give the patient time to benefit from emerging medical technologies such as antibody-based inhibition of oligomer formation [127].

Disclosure

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