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Review

Antibodies and protein misfolding: From structural research tools to therapeutic strategies☆

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ABSTRACT

Protein misfolding disorders, including the neurodegenerative conditions Alzheimer's disease (AD) and Parkinson's disease (PD) represent one of the major medical challenges or our time. The underlying molecular mechanisms that govern protein misfolding and its links with disease are very complex processes, involving the formation of transiently populated but highly toxic molecular species within the crowded environment of the cell and tissue. Nevertheless, much progress has been made in understanding these events in recent years through innovative experiments and therapeutic strategies, and in this review we present an overview of the key roles of antibodies and antibody fragments in these endeavors. We discuss in particular how these species are being used in combination with a variety of powerful biochemical and biophysical methodologies, including a range of spectroscopic and microscopic techniques applied not just *in vitro* but also *in situ* and *in vivo*, both to gain a better understanding of the mechanistic nature of protein misfolding and aggregation and also to design novel therapeutic strategies to combat the family of diseases with which they are associated. This article is part of a Special Issue entitled: Recent advances in molecular engineering of antibody.

1. Protein misfolding, amyloid deposition and disease

A wide range of human pathologies, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Creutzfeld Jacob disease (CJD), type II diabetes, amytrophic lateral sclerosis (ALS) and a variety of systemic amyloidoses are linked to the conversion of disease-specific proteins from their soluble state into highly ordered β-sheet rich fibrillar amyloid structures [1–6]. These disorders, known variously as protein misfolding aggregation, conformational or deposition diseases, have major human and economic costs to society.

All these diseases are characterized by the conversion of a normally soluble and functional protein into insoluble and pathogenic protein deposits in a variety of organs or tissues. When these disorders were originally investigated, the observation that affected tissue could be stained with iodine, led to the misconception that the deposits were rich in starch, leading to their common name as amyloid (starch-like) deposits [7]. Amyloid deposits are, however, largely composed of protein molecules that have aggregated into fibrillar species rich in ordered β-sheet structure. Each disease is associated with one or more specific proteins, *e.g.*, Amyloid-β (Aβ) peptide or tau protein in Alzheimer's disease (AD), mutant forms of huntingtin (mHTT) in Huntington's disease (HD), α-synuclein (aSyn) in Parkinson's disease (PD), β2-microglobulin (β2m) in dialysis-related amyloidosis (DRA) and the human prion protein (hPrP) in Creutzfeld–Jacob Disease (CJD) [4].

1.1. Mechanism of the formation of protein amyloid fibrils

It is now well established that the ability to form amyloid structures is not limited to the small subset of proteins or peptides that are involved in disease, but that any protein can in principal adopt this structural state [8–11]. The relative propensity of different proteins to convert into the amyloid state vary significantly, however, and depends on the overall thermodynamic stability of the native state of the protein as well as on the kinetic accessibility of partially folded intermediates that initiate the amyloid formation cascade [11,12]. The latter factor determines the frequency at which such conformations are sampled by a protein and thus the rate at which the protein is able to form fibrils under given conditions. Changes in the stability of specific proteins by

Abbreviations: AD, Alzheimer's disease; PD, Parkinson's disease; HD, Huntington's disease; CJD, Creutzfeld–Jacob disease; mAb, monoclonal antibody; scFv, single-chain Fv; VH, variable domain of the heavy chain; VL, variable domain of the light chain; VHH, variable domain of a camelid heavy-chain antibody; Nb, nanobody; WT, wild-type; HuL, human lysozyme; aSyn, α-synuclein; aβ, amyloid-beta; HuPrP, human prion protein; β2m, β2-microglobulin; ROS, reactive oxygen species; HTT, huntingtin; mHTT, mutant huntingtin; NAC, non amyloid component; BBB, blood–brain barrier

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the addition of certain co-solvents, denaturants, or as a result of the presence of destabilizing mutations, may allow amyloidogenic species to be sampled more frequently, thereby initiating the amyloid fibril formation process more readily [11,12].

The molecular mechanisms of the amyloid formation cascade and its associated toxicity have been extensively investigated *in vitro* and *in vivo* for a number of different proteins [13–20]. These studies have led to the realization that the ready formation of the amyloid form of proteins involves several key steps. Initially, a protein species with a high propensity for self-association needs to be formed, for example as a result of the exposure of hydrophobic residues; such species can be partially unfolded intermediates populated on the folding or unfolding pathways of globular proteins, or partially folded aggregation-prone members of the ensemble of structures in intrinsically disordered proteins [20]. In subsequent steps, these aggregation-prone species self-assemble into a variety of oligomeric species that convert into highly organized mature fibrils through a series of complex steps, typically involving primary nucleation, conformational rearrangement, aggregate growth and fragmentation, and secondary nucleation through surface mediated catalysis [11]. The kinetics of such underlying microscopic process of protein amyloid formation can now be analyzed and dissected using well-defined simple reaction schemes, such as those outlined in Fig. 1 [21–23]. These models allow accurate determination of the microscopic rate constants of the various primary and secondary processes involved in a given aggregation reaction from appropriate experimental measurements, and in addition provide the opportunity for the design and screenings of potential drugs that can perturb the specific microscopic events [3].

The resulting fibrillar structures have “generic” features, such as a characteristic “cross-β” structure, which can be readily established through X-ray fiber diffraction studies, and the ability to bind dyes such as Congo Red or Thioflavin T [24]. The basis for these generic features of amyloid fibrils can be found in the fact that the atomic interactions that stabilize the β-sheet structures of fibrils are primarily mediated through backbone atoms of polypeptide chains [11]. In contrast, the structures of the native states of globular proteins are determined by specific side-chain interactions that are characteristic of their unique individual sequences [25,26]. Indeed, as mentioned above, the amyloid structure can be considered to be a generic fold, which is accessible in principle under appropriate conditions by all proteins regardless of their sequences, although the propensity to convert into this state can vary widely [26].

The toxic nature of the different misfolded species populated during fibril formation is still a matter of intense discussion. In some situations, for example, in the case of misfolding of lysozyme, which underlies a type of familial systemic amyloidosis [27], the fibril deposits themselves are likely to be the direct cause of the disease by inflicting physical damage to vital organs; a similar situation occurs with liver damage caused by deposition of α1-antitrypsin although in this case the deposits are not specifically amyloid like in nature [28].

In a large number of studies, however, it is the smaller, more mobile oligomeric species with high surface-to-volume ratios and high hydrophobicities that have been identified as the direct mediators of cell damage and cell death [17,29]. Although these small oligomeric structures might be the most direct toxic agents, however, the presence of larger fibrils can contribute significantly to the toxicity, as these structures can, in at least some cases, enhance dramatically the generation of toxic oligomers through surface catalyzed secondary nucleation (Fig. 1). Such secondary processes may be of great importance in the progression and spreading of Alzheimer's disease [3,23] and Parkinson's disease [30], and their existence further underscores the importance of designing therapeutic strategies that suppress the occurrence of specific microscopic steps in the overall aggregation process [11].

1.2. Disease pathology: the mechanisms of cytotoxicity

As mentioned above, it is generally believed that the precursors of fibrillar deposits, particularly small oligomeric species, are important causative agents for cellular toxicity in protein deposition diseases. The high surface-to-volume ratios and presence of exposed hydrophobic residues [31] in such pre-fibrillar species are very likely to trigger aberrant behavior as a result of inappropriate interactions with cellular components, such as transcription factors, receptors or membranes, which can lead to high oxidative stress, or stimulate apoptosis or other forms of cell death. The high propensity of oligomeric species to bind to membranes, attributable to their flexible, dynamic and unstable nature, has received a great deal of attention in the literature [32–35], and could lead to pore formation and membrane permeability, which can in turn might lead to loss of protein homeostasis and the appropriate regulation of signaling pathways.

These oligomeric species have also been found to interact inappropriately with a number of cell surface receptors. In the context of AD, for example oligomeric species of the Aβ-peptide have been reported to interact strongly with the prion protein (PrP) [36,37], with NMDA [38,39] receptors and the associated tyrosine kinase EphB2 [39] receptor, and a variety of other cell-surface receptors (reviewed in [40]). Such interactions could possibly lead to aberrant

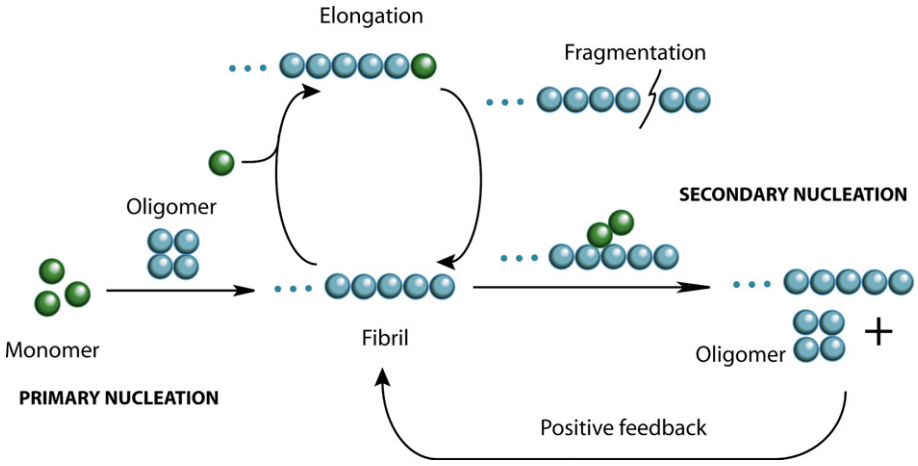

Fig. 1. A kinetic model of fibril formation including primary and secondary processes. Soluble monomeric forms of proteins undergo primary nucleation to generate oligomeric species that have the potential to convert into fibrils. The formation of fibrils is significantly enhanced by secondary processes that result in the proliferation of aggregates able to grow by the further addition of soluble protein molecules. The latter processes include fragmentation, which generates new fibril ends at which growth occurs and surface catalyzed nucleation, in which fibril surface functions as a template for the generation of new oligomeric species.
(Figure adapted from [22] and [23]).

signaling, hyper-phosphorylation of Tau (a protein associated with microtubule formation), and the production of reactive oxygen species (ROS) that then could eventually lead to synaptic dysfunction and apoptosis. Although different types of oligomeric species have been found to bind to these receptors, the identity and structural characteristics of the species responsible for the induced cytotoxic effects remains controversial [41]. Oligomers of the Aβ-peptide have also been shown to accumulate and exert cytotoxicity within the cytosol, such as proteosomal and mitochondrial dysfunction. Accumulation of Aβ oligomers possibly occurs through their interaction with the receptor for advanced glycation end products (RAGE) [42], the α7 nicotinic acetylcholine receptor α7nAChR [43,44] and/or the Apo-E [45,46] receptor.

In the case of HD, for which pathogenicity is thought to be initiated by the aggregation of mHTT within the cell, toxicity has been attributed to ER stress [47,48], mitochondrial dysfunction [49–51] and ROS production [52], and also to the loss of regulation of the N-methyl-D-aspartate receptor (NMDAR) [50] and of transcription [54] and nucleolar stress [53,54]. Also loss of function resulting from the direct sequestration of transcription factors, including transcription factors such as CBP [CREB (cAMP-response element binding protein)-binding protein] [55], TATA-binding protein [56] and Sin3a [57], by nuclear mHTT aggregates has been linked to the pathogenicity in HD. Also essential chaperones such as the low abundant Hsp40 chaperone Sis1p have been found to be sequestered by nuclear mHTT aggregates leading to the impairment of the UPS –mediated degradation of misfolded proteins [58]. In addition, it has been shown that enhanced mHTT aggregation into fibrillar structures can cause increased cell oxidative stress, suggesting that the fibrillar material itself could contribute directly to this toxic mechanism [52].

In Parkinson's disease oligomeric species have also been suggested as major culprits of neurodegeneration, including the direct observation of the formation of a specific type of oligomeric species of aSyn, using single molecule fluorescence techniques, that have been shown to be toxic [59]. The kinetics of oligomer formation were also shown to correlate strongly with increased ROS levels in cultures of primary neurons, in contrast with low levels of toxicity found for other oligomeric species and for the fibrils themselves [59]. In addition, as for the Aβ peptide associated with AD, oligomeric species of aSyn, have also been found to interact strongly with cellular membranes leading to membrane permeabilization and interference with ionic-homeostasis [60,61]. Furthermore, as with Aβ oligomers, oligomeric species of aSyn have also been shown to interact aberrantly with α4β2 nicotinic acetylcholine receptors [62]. Additionally it has been shown that aSyn oligomers activate microglia *via* the activation of Toll-like receptor 2 (TLR2), inducing neuroinflammation and leading to enhanced neurodegeneration [63].

2. Generation of antibodies and antibody fragments

The high specificity and binding affinity of antibodies have made these species invaluable and unique tools for a vast range of biotechnological and pharmaceutical applications. Before the development of hybridoma technology [64], antibodies had to be produced as polyclonal anti-sera in immunized animals, but there were already many different applications, including passive immunizations for therapeutic purposes pioneered by von Behring and Kitasato [65].

Hybridoma technology [64] revolutionized the field as it allowed relatively easy production of monoclonal antibodies and the possibility of their manipulation and modification, including the generation of bispecific constructs and humanization [66]. More recently, phage-display [66,67], ribosome display [68,69] and yeast-display [70,71] technologies have further impacted the field significantly by allowing antibody fragments, including Fab and scFv domains and also nanobodies, see below, to be selected from synthetic and naïve libraries, effectively bypassing the immune system [72]. The microbial expression systems allow easy and cheap production of specific antibody fragments and efficient protein engineering, including *in vitro* affinity maturation and the generation of manifold constructs containing new functionalities [72].

Antibody fragments, as opposed to full-length antibodies, are indeed very attractive in many biotechnological and clinical applications that do not require the effector functions of the complete antibody molecule, as they are much smaller and better expressed [73]. Moreover, these fragments are also often found to express and function well and inside the cell allowing intracellular bio-molecules to be targeted; the antibody fragment is then referred to as an intrabody (intra-cellular antibody fragments) [74,75]. Antibody fragments are derived from full-length conventional antibodies and can be composed of the separate VH [76–78] or VL [78,79] domain of the complete antibody (single-domain antibodies) or, more commonly, of both domains genetically linked by a flexbile Gly-Ser linker (single-chain Fv, scFv) [80]. Nanobodies are an additional important type of fragment, which have been developed following the very surprising discovery of naturally occurring antibodies in camelids that lack the light chains of conventional antibodies (Fig. 2) [81,82]. Nanobodies are the single binding domains of camelid heavy chain antibodies (VHH) and have comparable binding strengths and specificities to their conventional Fv equivalents, but they are superior in stability and solubility, allowing easy production and manipulation [83–85]. For these reasons, nanobodies are now rapidly emerging to become very strong competitors of conventional antibodies and antibody fragments in biotechnology [86].

2.1. Antibodies targeted against amyloidogenic proteins

A wide range of antibodies, either monoclonals, or fragments obtained from phage display libraries, has been generated against a variety of proteins and peptides involved in protein misfolding diseases. These include scFv's, nanobodies and VL domains and have been designed to be used for basic immunohistochemistry, rational drug design, and disease target validation and to explore potential therapeutic strategies. The field is very extensive and so in this article we will limit ourselves to the review of studies in which antibodies have been central to the elucidation of key aspects of the mechanisms of amyloid formation and toxicity for a limited number of systems, including those relating to AD (Aβ), PD and other synucleinopathies (aSyn), HD (mHTT), CJD and other prion protein related diseases (PrP), dialysis related amyloidosis and systemic amyloidosis (HuL) (Table 1). In many cases, the antibodies were raised against the native monomeric form of the amyloidogenic protein [87–92], although some polyclonals, monoclonals and antibody fragments have been raised against oligomeric or fibrillar forms of Aβ and aSyn (see Table 1), either through immunization or through the creation of synthetic phage-display libraries [93–99].

3. Structural and mechanistic studies of amyloid formation using antibodies

3.1. Mechanistic insights into amyloid formation in vitro

Antibody fragments can be highly informative about the nature of the different species formed along the aggregation pathway of an amyloidogenic protein. Epitope accessibility and conformational changes typically have dramatic effects on antibody binding interactions, and thermodynamic analysis can offer indirect insights into the nature of structural changes [124,125]. Antibodies that bind to different regions of a protein molecule allow mechanistic insights into the structural role of these epitopes in the processes associated with amyloid formation.

Studies of the amyloid formation by HuL illustrate this point particularly clearly. HuL fibril formation is initiated by the cooperative unfolding of the β-domain of the protein [126], which can then associate with other partially unfolded monomers to form oligomeric, prefibrillar and fibrillar species (Fig. 3). This cooperative unfolding of the β-domain is enhanced

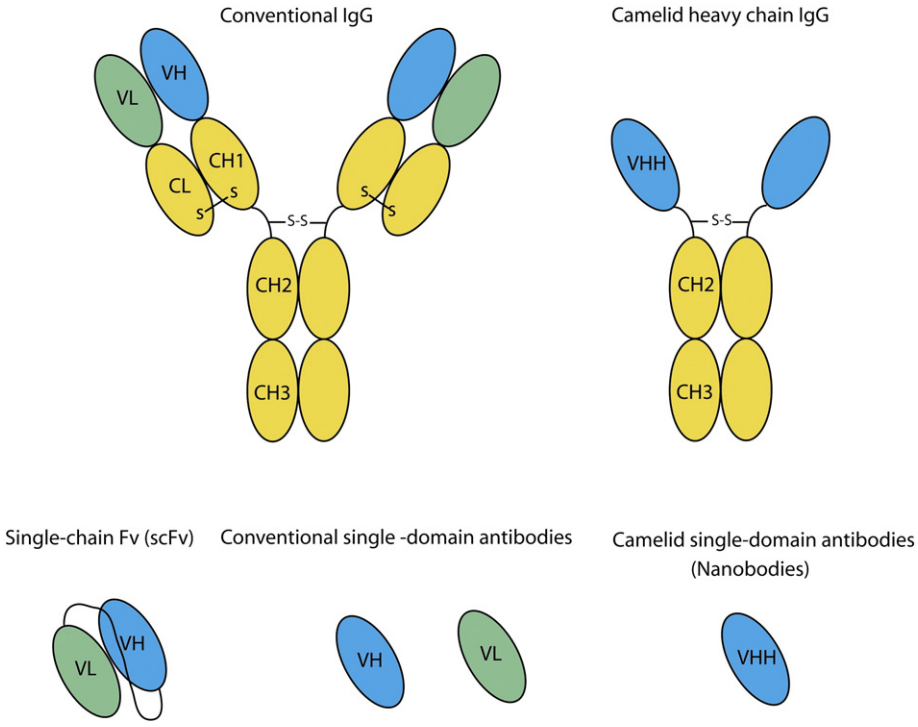

Fig. 2. Conventional and camelid (heavy-chain) antibodies and the antigen-binding fragments derived from these.

by disease related hereditary mutations that are located in this domain or in the α/β interface of the protein [27,127], which reduce the overall stability of the protein as well as its global cooperativity [128]. A series of nanobodies raised against lysozyme has been found to bind to different epitopes on the protein [87–89,100], (Table 1) and all of these were found to inhibit the formation of fibrils, regardless of the epitope concerned. It has however been shown that only the nanobodies that bind simultaneously to both the α — and β — domains of the protein were capable of preventing the cooperative unfolding of the β-domain [128] The nanobody, cAb-Hul5, however, was found to bind to the α-domain of the protein and revealed that this domain is required to undergo a further unfolding step in order to allow this region of the sequence to be incorporated into a fibrillar structure (Fig. 3) [100].

For α-synuclein in contrast we have shown that nanobodies raised against the C-terminal residues of the protein (Table 1) do not influence dramatically the kinetics of fibril formation by α-synuclein but that binding data in combination with thermodynamic analysis can be very informative for deciphering the subtle structural transitions that take place during aSyn fibril maturation [90,101].

In a different study, a scFv with high affinity for monomeric aSyn was isolated from a synthetic human scFv library and shown to bind to two different regions in the aSyn sequence, including residues 27–37 in the N-terminal region and residues 101–111 in the C-terminal region of the protein, but with a preference for residues 101–111 [99]. This observed cross-reactivity of the scFv with different regions on the protein is most probably due to a shared –GKxxEG- motif in both epitopes. It was found that the antibody fragment also bound to the fibrils of aSyn, which suggests that a similar motif, either in N-terminal region or the C-terminal region must be exposed to the solvent [99].

3.2. Structural studies of kinetically trapped amyloidogenic intermediates

Early events on the amyloid formation pathway of any protein are likely to be extremely hard to characterize as they typically represent high-energy states of the system, and hence are very low in population, and can convert directly into the highly stable amyloid fibril state [11]. Obtaining any structural information on these events is therefore a major challenge using conventional techniques, such as X-ray crystallography and NMR spectroscopy, which rely on the presence of homogeneous and well-populated protein species. Antibody binding can serve to trap species that are present only at low populations, sometimes enabling the crystallization of the complex to provide clues as to the nature of the intermediate species.

Indeed for the proteins β2m [123] and HuPrP^{c} [121], nanobodies were used in this way and have shed light on the possible mechanisms by which nucleation of aggregate formation would occur. Domanska et al. [123], crystallised a complex between a nanobody, Nb24 (Table 1), and the amyloidogenic N-terminal truncated variant of β2m, ΔN6β_2m, a protein involved in dialysis related amyloidosis (DRA), a condition that occurs in patients that undergo prolonged haemodyalis, which causes a gradual build up β_2m protein due to ineffecient permeability of the dialysis membrane [129]. In the crystal lattice, it was observed that two Nb24:ΔN6β2m complexes were bonded to each other to form a hetero tetramer, and which was the result of the structural exchange of the C-terminal ß-stands of two ΔN6β2m molecules. This observation led to the suggestion that a domain swapped species could be an intermediate species in the formation of β-sheet rich fibrils of β_2m [123]. This structure offers important insights into the mechanism by which β2m forms amyloid fibrils, and the structural details observed in this study are consistent with previously published and more recent work on the possible determinants of β2m fibril formation [130–136].

In the case of HuPrP^{C} the crystal structure of the full-length protein in complex with the Nb484 nanobody shows how the palindromic motif AGAAAAGA in the HuPrP, which is unstructured in solution, forms an extended β-strand that engages with the B1 strands of the core of the protein to make an extended β-sheet [121]. This palindromic sequence is located in the generally disordered N-terminal region of HuPrP and is believed to play a central role in the conversion of soluble HuPrP^{C} into the aggregated and potentially pathogenic form of the protein HuPrP^{Sc}.

In a study of the mechanism of the aggregation of Aβ, a peptide containing the sequence 18–41 (equivalent to the p3 α/γ-secretase fragment of the precursor protein APP), was incorporated within the CDR3 loop of a (shark Ig new antigen receptor (IgNAR)) single variable

Table 1
Generation of antibody fragments against amyloidogenic protein targets.

Antibody	Type/isolation method	Target protein/antigen	Species/epitope	Reference
cAb-HuL6	Nanobody/immunization & phage-display	HuL/WT HuL	Monomer/α/β domain and C-helix, residues 15, 16, 20, 76–79 90, 91, 94, 95, 97, 98, 101	[88,89]
cAb-Hul22	Nanobody/immunization & phage-display	HuL/WT HuL	Monomer/α/β domain	[87]
cAb-Hul5	Nanobody/immunization & phage-display	HuL/WT HuL	Monomer/α domain, residues 10, 13, 14, 16, 18, 19, 22–24, 26, 27, 121, 122, 130	[100]
NbSyn2	Nanobody/immunization & phage-display	aSyn/human WT aSyn	Monomer/residues 135–140	[90]
NbSyn87	Nanobody/immunization & phage-display	aSyn/human aSyn A53T	Monomer/residues 118–132	[101]
Syn-10H	scFv/synthetic human scFv library & phage display	aSyn/human WT aSyn (oligomer and fibrillar form)	Oligomer and SDS stable trimer or hexamer/n.d.[a]	[52,102]
D10	scFv/synthetic human scFv library & phage display	aSyn/WT aSyn	Monomer/residues 27–37 and 110–121	[99,103]
D5	scFv/synthetic human scFv library & phage display	aSyn/WT aSyn	Oligomer and SDS stable dimer or tetramer/n.d.[a]	[104]
NAC32	scFv/synthetic human scFv library & yeast display	aSyn/$aSyn_{53-87}$-biotin	Monomer/NAC region residues 53–87	[105]
VH14	VH/synthetic human scFv library & yeast display	aSyn/$aSyn_{53-87}$-biotin	Monomer/NAC region, residues 53–87	[105,106]
C1	scFv/synthetic human scFv library & phage display	Aβ/$Aβ_{1-40}$	Monomer/residues 29–40	[107]
H1v2	scFv/synthetic human scFv library & phage display	Aβ/$Aβ_{1-28}$	Monomer/residues 17–28	[107]
B6 & D4	scFv/synthetic human scFv library & phage display	Aβ/$Aβ_{25-35}$	Monomer/residues 25–35	[108]
B10	Nanobody/synthetic nanobody library & phage-display	Aβ/$Aβ_{1-40}$ fibrils	Fibrils/n.d.[a]	[98]
E1	scFv/synthetic human scFv library & phage display	Aβ/$Aβ_{1-42}$ oligomers	Small oligomers/n.d.[a]	[109,110]
A4	scFv/synthetic human scFv library & phage display	Aβ/$Aβ_{1-42}$ oligomers	Oligomers/n.d.[a]	[109,110]
C6	scFv/synthetic human scFv library & phage display	Aβ/brain-derived Aβ oligomers	Oligomers (from brain)/n.d.[a]	[96]
A11	Polyclonal IgG/rabbit immunization	Aβ/$Aβ_{1-42}$-coated gold particles	Oligomers (pan-reactive)/n.d.[a]	[111,112]
OC	Polyclonal IgG/rabbit immunization	Aβ/$Aβ_{1-42}$ fibrils	Fibril (pan-reactive)/n.d.[c]	[112]
Happ 1&3	VL/synthetic human scFv library & phage display	HTTexon1/polyP-PQLPQPPPQAQP-PolyP	Monomer/proline rich region	[113]
scFvC4	scFv/synthetic human scFv library & phage display	HTTexon1/$HTTexon1_{1-17}$-biotin	Monomer/residues 5–17	[114]
VL12.3	VL/synthetic human scFv library & yeast display	HTTexon1/$HTTexon1_{1-17}$-biotin	Monomer/residues 1–20	[115,116]
MW1	mAb & scFv/mouse immunization & hybridoma	HTTexon1/GST-$DRPLA_{1-35}$-Q19[b]	Monomer/polyQ region	[117,118]
MW2	mAb & scFv/mouse immunization & hybridoma	HTTexon1/GST-$DRPLA_{1-35}$-Q19[b]	Monomer/polyQ region	[117,118]
MW5	mAb & scFv/mouse immunization & hybridoma	HTTexon1/GST-$DRPLA_{1-35}$-Q35[b]	Monomer/polyQ region	[117,118]
MW3,4 & 6	mAb/mouse immunization & hybridoma	HTTexon1/mHTTexon1-Q67	Monomer/polyQ region	[117]
MW7	mAb & scFv/mouse immunization & hybridoma	HTTexon1/mHTTexon1-Q96 (+boost with fibrils)	Monomer/proline rich region	[117,118]
MW8	mAb & scFv/mouse immunization & hybridoma	HTTexon1/mHTTexon1-Q96 (+boost with fibrils)	mHTT inclusions/8 last residues mHTTexon1	[117]
EM48	scFv/rabbit immunization & hybridoma	HTTexon1/GST-HTT_{1-250}	Monomer/HTTexon1 C-terminal region	[119,120]
Nb484	Nanobody/immunization & phage display	PrP/rec PrP_{23-231}[c]	Monomer/residues 123 − 125,164 − 170,174 − 185	[121]
POM2& 12	mAb & scFv/mouse immunization & hybridoma	PrP/rec PrP_{23-231}[c]	Monomer/residues 57–64, 64–72, 72–80, 80–88	[122]
POM11	mAb/mouse immunization & hybridoma	PrP/rec PrP_{23-231}[c]	Monomer/residues 64–72, 72–80	[122]
POM3	mAb/mouse immunization & hybridoma	PrP/rec PrP_{23-231}[c]	Monomer/residues 95–100	[122]
POM5	mAb/mouse immunization & hybridoma	PrP/rec PrP_{23-231}[c]	Monomer/β2–α2 loop, α2 residues 168–174	[122]
POM4,10& 19	mAb/mouse immunization & hybridoma	PrP/rec PrP_{23-231}[c]	Monomer/β1 and α3, residues 121–134 and 218–221	[122]
POM1	mAb/mouse immunization & hybridoma	PrP/rec PrP_{23-231}[c]	Monomer/β1–α1 loop, α1 and α3 138–147; residues 204/208/212	[122]
POM6,7,13,15 & 17	mAb/mouse immunization & hybridoma	PrP/rec PrP_{23-231}[c]	Monomer/β1–α1 loop, α1 β2–α2 loop, α2 residues 140/145; 158/177; 170/174	[122]
POM8 & 9	mAb/mouse immunization & hybridoma	PrP/rec PrP_{23-231}[c]	Monomer/β1–α1 loop, α1 β2–α2 loop, α2 residues140/145; 170/174	[122]
Nb24	Nanobody/immunization & phage display	$β_2$m	Monomer residues 41–45, 75–77	[123]

[a] n.d., not determined.
[b] DRPLA, dentatorubral palliodoluysian atrophy.
[c] rec, recombinant.

domain antibody. Such chimeras form oligomeric species as a result of interactions between inserted regions of Aβ with paired dimers forming a tetramer in the crystal caged within four IgNAR domains, inhibiting further assembly into amyloid fibrils [137]. The structure provided a possible structural mechanism for $Aβ_{17-42}$ oligomerization, involving a tight arrangement of the amyloidogenic peptide into a non-cross β-sheet tetrameric arrangement [137].

3.3. Stabilization of transiently populated oligomeric states by antibodies

As the oligomeric precursors of amyloid fibrils are widely considered to be the most toxic species generated during the aggregation of peptides and proteins, considerable effort is expended in their characterization. This objective is, however, extremely challenging because of the heterogeneous and transient nature of these assemblies. Moreover, the peptides or proteins associated with amyloid diseases are often intrinsically disordered, as is the case for the Aβ-peptides in AD and aSyn in PD [11]. Such proteins typically adopt highly diverse and rapidly inter-converting conformational states that make the use of conventional techniques, such as X-ray crystallography and NMR spectroscopy, for the structural characterization or even the soluble precursor states particularly demanding, although NMR techniques have made it possible to define detailed ensembles that are representative of these states [138]. The development of structural approaches that are highly sensitive to structural motifs that are transiently populated are therefore of great significance for investigating such species, along with the variety of aggregated assemblies that are formed during the process of fibril formation [124]. Indeed, given the crucial role that the small highly

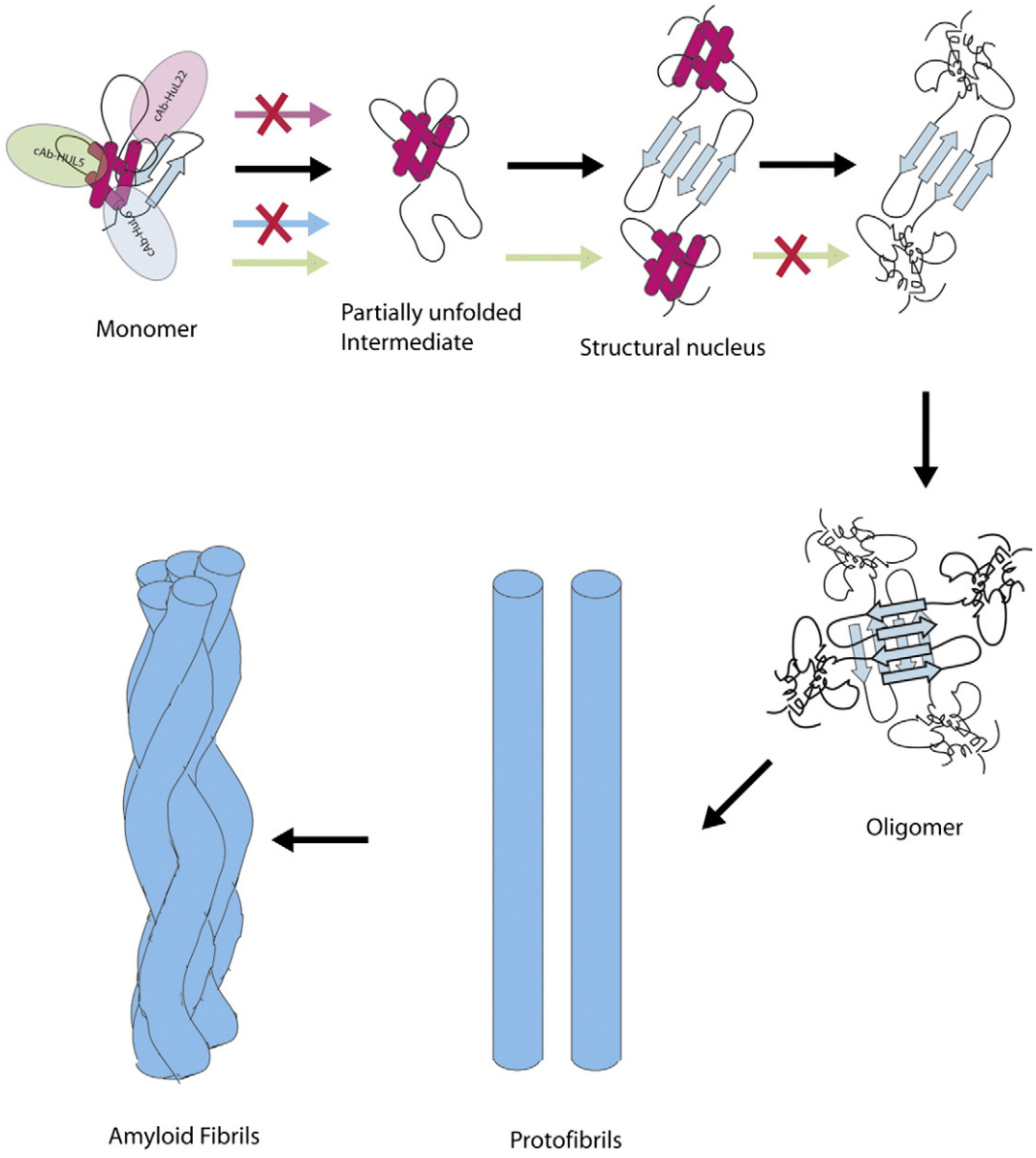

Fig. 3. Schematic description of the process of amyloid formation by human lysozyme and the effects of lysozyme specific conformational antibody fragments. (Adapted from [124] and [100]).

toxic oligomers play in the underlying pathology of misfolding diseases, it is of particular importance to obtain detailed structural information on these specific species, in order to increase our understanding of the underlying structural and physical aspects of their formation, and of the origin of their pathogenic nature.

The assumption of such information will be of key significance for the design of potential therapeutics based on the strategies of suppression of their formation or the reduction in their toxicity. At present we know relatively little about the detailed molecular structures of these crucial species, and a wide variety of oligomeric assemblies that differ significantly in size and morphology can often be generated under different conditions for any given protein. There is, therefore, a need for a stringent classification system for the different oligomeric species to define clearly the origin of specific cytotoxic effects [139]. The high specificity and high affinity of antibodies for their antigens, could, therefore, enable them to play a key role in this objective, as selective and extremely sensitive probes that can discriminate between oligomers of different size and structure [140–142]. Antibodies can, in addition, stabilize or solubilize certain forms of aggregates, and thereby potentially allow them to be studied by well-established structural techniques. Thus, for example, antibodies have been used very successfully to solubilize and crystallize membrane proteins; important examples are studies of the structures of a series of various G protein-coupled receptors [143–145].

A number of strategies have been used to generate antibodies against toxic oligomeric species. Of particular interest have been the recent reports involving the immunization of mice with gold particles coated with Aβ, mimicking an oligomeric form of the peptide [139, 146–148], that resulted in the polyclonal antibody, A11, that has been found to cross-react with protein oligomeric species formed from other types of peptides and proteins (including aSyn, HTT, SOD, amyloid light chain). These findings suggest that a generic conformational epitope exists rather than one characterized by specific amino acid side-chains [111]. The polyclonal A11 antibody (Table 1) that interacts with the oligomers does not bind to the mature fibrillar form of the antigens, or indeed to their soluble states consistent with the hypothesis that major structural transitions are associated with the formation of these oligomers and that they undergo further major structural rearrangements upon fibril formation [111].

Using a similar immunization strategy, but based on Aβ fibrils, polyclonal antibodies, denoted OC (Table 1), were obtained, which interact specifically with fibrillar forms of several proteins but do not interact with oligomeric pre-fibrillar species or with monomeric Aβ [112]. Both the A11 and the OC antibodies have been widely used to distinguish between oligomeric and fibrillar species of a range of different proteins [139].

A combination phage-display and atomic force microscopy has been used as an alternative strategy that has enabled isolation of scFv fragments from a human synthetic scFv library that bind to visualized oligomeric or fibrillar forms of Aβ and/or αSyn, with variable cross-reactivity to other proteins [97,102,104,109,149]. It was shown that substiochiometric quantities of these anti-oligomeric scFv's were able to inhibit the formation of aSyn fibrils, and also to suppress dramatically the cellular toxicity of synthetically formed oligomeric species of aSyn. A scFv against fibrillar aSyn, however, cross-reacted with fibrils formed by mutant huntingtin (mHTTexon1) fragments. In this case, co-expression of the aggregating mutant protein with the anti-fibillar scFv as an intrabody increased both the size of the intracellular aggregates and cytotoxicity [52], and illustrates that antibody fragments targeting toxic species of proteins can either increase or decrease toxicity.

A different approach using VH single domain antibodies against oligomeric and fibrillar forms of peptides and proteins has also been developed and applied to Aβ, aSyn and islet amyloid polypeptide (IAPP) [93,94]. Here the VH domain antibodies have been synthetically designed to contain short sequences of residues from regions of the targeted proteins that are expected to be highly amyloidogenic. These segments are then inserted genetically into the CDR3 loop of the VH domain antibodies, and depending on the length of the sequence that is inserted, antibodies binding to either to oligomers or fibrils is observed. The resulting VH domain antibodies have been found to inhibit effectively formation of fibrils by the proteins that were targeted in this way, and have the capacity to reduce the toxicity associated with the oligomers[93,94].

4. *In situ* and *in vivo* studies of protein misfolding using antibodies and antibody fragments

Protein aggregation and its underlying toxic effects occur in a very complex environment *in vivo*, involving many different components, including biological membranes, membrane bound receptors, molecular chaperones and many other biomolecules [11,150,151]. Amyloid formation is often thought to initiate within the cell, but it can then be spread to the extracellular space and to neighboring cells [152,153]. Aggregation behavior of the peptides and proteins that have been measured *in vitro*, and the consequent effects of this process, such as toxicity, must therefore be translated into the analogous behavior in a cellular environment and indeed in a living organism. In addition, potential drugs discovered or developed *in vitro*, including antibodies and antibody fragments, must be examined to ensure that they are influencing the aggregation behavior *in vivo* as well as *in vitro*. In the next section we discuss a number of systems for which antibodies have been used both to probe the mechanism of aggregation and to explore the possibility of their use as therapeutics.

4.1. Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder, characterized by severe motor and variable psychiatric dysfunctions [154]. The underlying molecular origin of HD involves an aberrant expansion of the N-terminal polyglutamine (polyQ) tracts of the protein huntingtin (HTT) [155]. HTT is found predominantly in the cytosol, but in HD, N-terminal proteolytic fragments encompassing the first exon of mutant forms of HTT (mHTTexon1), which are characterized by aberrant expansions of the wild-type polyQ tract (on average 16–20 glutamines) to a polyQ tract that exceeds 36 and up to 250 glutamines in mHTT, with an inverse correlation of both onset of motor symptoms and age of death with the polyQ repeat length in HD subjects [155–157]. These mHTTexon1 fragments are found to accumulate as insoluble inclusions in cellular nuclei [158,159]. A number of antibodies and antibody fragments that bind to mHTTexon1 have been isolated, some of which can be expressed within different cell-types, including neuroblastoma, human embryonic kidney or striatal cells as soluble intrabodies (Table 1) [52,99,113–115,117,118,160–162]. It is now apparent that the ability of anti-mHTTexon1 intrabodies to modulate aggregation and its associated neurotoxicity strongly depends on the epitope of HTT toward which they are directed [163].

A 17-residue sequence denoted HTT(1–17) is located immediately N-terminal to the polyQ tract in HTT, and has been shown to be involved in membrane binding and sub-cellular localization, as well as being associated with aggregation and toxicity [164–172]. The sequences flanking polyQ at its C- and N-terminal region have opposite effects on the kinetics of the aggregation of mHTTexon1 fragments when studied *in vitro* [173]. The key role of the HTT(1–17) sequence in the pathogenic deposition of mHTT aggregates and its inhibition by molecular chaperones that bind to this region [174] make it a very attractive target for the development of intrabodies able to suppress mHTTexon1 aggregation [163]. Two intrabodies have so far been reported that bind to this region, VL12.3 [115,116,175] and scFvC4 [114,160], and both antibody fragments have been found to bind to the N-terminal residues of HTTexon1 preceding the polyQ region and to reduce very potently mHTTexon1-induced aggregation and toxicity *in vitro* and *in situ*, while not altering significantly the turnover of mHTT [73,114,160]. The VL12.3 appears, however, to block the retention of mHTTexon1 in the cytoplasm, and indeed to give rise to much higher levels of the antigen-antibody complex in the nucleus,[113,176] a compartment that is known to be a major site of mHTT pathogenesis. When VL12.3 is delivered directly to HTT transgenic mouse brains *via* gene therapy, there is also a slight increase in disease severity [176]. By contrast, the complexes of scFvC4 and mHTTexon1 show significant localization in the cytoplasm, and reduce the aggregation phenotype when delivered as genes ([177] and Butler *et al*, in prep). To counteract the long-term kinetic effects of aggregation *in vivo*, scFvC4 was engineered to include a fusion to a proteosomal degradation sequence, with enhanced protective effects *in situ* [178]. These studies therefore underscore the importance of validation of specific targets in a cellular context.

In a study that was aimed at developing immunohistochemistry reagents for HD pathology, eight monoclonal antibodies, MW1–8, binding to the polyQ tract or to the C-terminal regions of mHTTexon1 were generated [117]. The mAbs MW1–6, were obtained by immunizing Balb/c mice using a number of soluble GST fusion proteins containing polyQ sequences of different lengths. The mAbs MW7–8 were obtained using fibrillar forms of mHTTexon1 in the boosting steps of the immunization [117,179]. The mAbs MW1–6 were found to bind to the polyQ region while the mAbs MW7 and MW8, were found to bind to the poly-proline region and the eight last residues in the C-terminal region of mHTTexon1, respectively. Remarkably, Western blot analysis and epitope mapping revealed that MW1–6 were found to bind preferentially to the polyQ region of the expanded repeat (mutant) form of HTT, in contrast to that of the wild-type HTT protein, very similar to the observations made for two other polyQ binding mAbs 1C2 and 1F8 [117,180,181]. Furthermore, it was found that MW1–6 bind to polyQ in a linear conformation, consistent with the extended conformation of the peptide $GQ_{10}G$ observed in the crystal structure of the MW1:peptide complex [182]. Further analysis revealed that MW1–6 poorly stained nuclear inclusions HD mouse brain sections, in contrast to the mAbs MW7 and MW8. In addition, remarkable differences in the staining patterns between sub-cellular compartments of HD mouse brain sections as well as between HD mice and healthy controls led to the suggestion that different regions of mHTTexon1 have different solvent accessibilities, either due to the binding of other sub-cellular specific proteins or due to conformational changes of the protein. In a second

study, the variable domains of MW1 and 2 were recloned into an scFv format and assessed for their ability to reduce mHTT cytotoxicity in human embryonic kidney cells, HEK293 [118]. Very interestingly, it was found that scFv MW1 and scFv MW2 enhanced mHTT aggregation and cell-death, results that can be explained by the fact that the antibody has a preference for binding to longer and a toxic expanded polyQ conformation [118]. These results are also consistent with the effect of a pan-specific anti-fibrillar scFv, scFv-6E, that also enhances aggregation of mHTT and increased the probability of induced cell-death in Huntington's disease models [52].

Several antibodies were raised against the C-terminal proline-rich region of mHTTexon1 [113,117]. This region is believed to be involved in a number of functions, including endocytosis [183] and interactions with various transcription factors, including the WW domain proteins HYPB and HYPC and the splicesome FBP-11 [183], as well as IKKγ, a regulatory subunit of the IκB kinase complex, and the cell-cycle regulatory protein P53 [184]. This C-terminal proline-rich region also has a strong modulating effect on the aggregation of mHTTexon1 *in vitro* [185]. The fragments of the antibodies that were raised against this region include the mAb MW7 ad its scFv variant [117]; and the VL domains Happ1 and Happ3, which were all successfully expressed as intrabodies [113]. The intrabodies Happ1 and Happ3 were also found to very strongly inhibit the aggregation and toxicity of mHTTexon1, and to increase significantly the turnover of the protein, suggesting that the proline-rich region is important for the stability of the protein [113].

4.2. Parkinson's disease

PD is the second most common neurodegenerative disorder affecting approximately 1% of the population over the age of 65 [186]. Clinical symptoms of PD include resting tremor, slowness of movement, muscular rigidity and impairment of postural reflex [187]. PD is characterized neuropathologically by Lewy bodies and Lewy neuritis, which are intracellular inclusions containing fibrils of human aSyn, an intrinsically disordered, pre-synaptic 140-residue protein that is involved in vesicular [176] transport and neurotransmitter release [188,189]. There is also genetic evidence implicating aSyn in the pathogenesis of PD, with three point mutations (A30P, E46K and A53T) and gene triplication of aSyn known to cause dominantly inherited early onset PD, and risk factor polymorphisms in the promoter [188–190]. Despite the clear presence of aSyn fibrils in the brains of PD patients, it is becoming increasingly evident, as in other neurodegenerative conditions, that these fibrils are not the most toxic forms of the aggregated protein, but that instead smaller oligomers are likely to be the more damaging species [59,191,192].

As with HD, PD is linked to amyloid formation of aSyn that occurs at least initially inside the cell, and at later stages can spread to neighboring cells [193]. Cellular, *Drosophila* and animal models, overexpressing aSyn, intracellularly accumulate aSyn inclusions and other PD pathology and various intrabodies targeting aSyn have been evaluated using *in situ* models. The intrabody scFv D10 that targets monomeric aSyn and has been shown to bind intracellularly to aSyn, increasing the soluble fraction of the protein and ameliorating the cell adhesion properties that are impaired as a result of over-expression and aggregation of aSyn [103]. In addition, the human VH domain, VH14, and the scFv, NAC32 were selected from a yeast surface display library to bind the relatively hydrophobic, amyloidogenic region of aSyn, also known as the NAC region, which is critical for aSyn aggregation (Table 1) [105]. NAC32 was reasonably soluble as an intrabody, and was able to restore cell viability and reduce significantly the cytoxicity by the overexpression the A53T disease related variant of aSyn in rat neuronal cell lines [105]. The modification of VH14 as a genetic fusion with the PEST proteasomal degradation signal of mouse ornthine decarboxylase, has been shown to enhance the efficacy of the intrabody further, a finding attributable to the combined effects of the increased solubility of the intrabody and the increased turnover of aSyn resulting from intrabody binding [106].

5. Antibody based therapeutic strategies

The major challenge for antibody-based therapies directed at diseases of the central nervous system (CNS) remains the presence of the physiological protection of the blood brain barrier (BBB), which limits the accessibility of most macromolecules to the brain [194]. Some nonspecific permeability does, however, exist and can be exploited by using high serum antibody concentrations, which may also serve as a peripheral sink for the aberrant protein species. Alternatively, antibodies can be engineered to incorporate sequences that have naturally occurring BBB permeability, or they can be engineered to cross this barrier through active transport either by direct binding to an endothelial receptor or through fusion to proteins that bind to specific receptors for the transport of molecules through the endothelial barrier [195–198].

Direct delivery of antibodies within the brain or spinal cord is a more invasive but potentially more powerful approach that can be achieved *via* injections, pumps, or delivery as genes *via* viral vectors [199–202]. Antibodies are likely to be effective in dealing with extracellular proteins, including those species that would be responsible for the spread of pathogenesis through cell-to-cell transmission [193]. There is, however, evidence from both paraneoplastic neurodegeneration [203,204] and clearance of intra-neuronal viruses without causing damage to neurons that native antibodies can act intracellularly [205]. Genes encoding binding fragments can also be delivered directly to neurons, with the protein products acting as intrabodies [5].

AD has been the main focus for immunotherapy for a number of years [206,207], using both active [208,209] and passive [210] immunization strategies and some preclinical studies for Parkinson's disease have also been explored [211–214], and very recently a phase I clinical trial has been started for the anti-aSyn mAb PRX002 in the US (https://clinicaltrials.gov/ct2/show/study/NCT02157714).

Whereas active immunization held great promise for AD originally, clinical trials failed to show significant improvement in cognition and mortality, and presented a great risk of inducing severe inflammation of brain, exacerbating the disease and leading to increased mortality [207,215,216]. The passive immunization strategies using monoclonal antibodies have also been extensively explored, with its best-known candidates, bapineuzumab [217] (manufactured by ELAN) and solanezumab [218] (manufactured by Lili). Clinical trials using these antibodies, however, showed little or no cognitive efficacy [207], possibly due to the fact that neurodegeneration was already quite advanced in the patients well before the start of immunotherapy [212]. The fact that patients with mild forms of the disease did seem to receive modest benefits of the immunotherapy with bapineuzumab, shifted focus and efforts towards treating AD prior to or in the earliest stages of neurodegeneration, however recent phase III clinical trials of immunotherapy with both bapineuzumab and solanezumab failed to reveal any significant improvements in cognitive functions for patients with mild forms of AD [219,220].

6. Conclusions and future directions

Recent studies are beginning to reveal structural characteristics of the mechanisms by which soluble peptides and proteins convert into amyloid fibrils including the nature of the variety of oligomeric species, populated in such processes, including their roles in toxicity, the manner in which they interact with receptors and membranes. Such information provides clues as to possible ways of preventing this aberrant behavior, perhaps through the discovery of molecules that perturb individual steps in the mechanism of aggregation. As we discuss here in this article it is very likely that antibodies will play a key role in such studies. Their high specificity and affinity make them very selective

and sensitive probes that can detect transient species and conformations, within a mixture of different protein conformers of the aggregating protein. Moreover, the development of robust antibody fragments with enhanced functional expression within cells will not only allow these antibody fragments to be used as research tools and diagnostics, but will also facilitate their development as powerful therapeutics.

Although antibodies have a very high potential to be used as therapeutics for protein misfolding diseases, careful target validation for intervention is clearly needed, and oligomeric species are attractive candidates, because the protein in its native state should be able to carry out its normal function. The stabilization of oligomeric species by binding to the antibody, however, might enhance their formation, and might be a dangerous strategy to follow. Furthermore, major hurdles will need to be overcome in delivery strategies, not least when the blood brain barrier needs to be crossed to targets that reside in the cell. These strategies will most probably be disease dependent as the toxic function of the protein species formed will be dependent on the interplay of its levels in the specific tissue and on its exact location outside or within the cell.

Also the timing of intervention will be important. The major cause of neurodegeneration is sporadic and age related and clear outward symptoms appear only very late in the disease progression. For this reason early diagnosis of neurodegenerative conditions is very difficult, which possibly has contributed to the recent failures of active and passive immunization strategies to halt disease progression and death, as the research subjects might have been already in a too advanced stage of the disease for the therapy to be effective. Therefore biomarker development as well as the development of preferably non-invasive methodology for early detection of disease onset would be highly beneficial for the development of effective treatments. Also in this area, antibodies will probably play a key role as the development of good diagnostic methods, able to detect disease specific pathogenic species will most probably require highly sensitive reagents.

In conclusion, it is clear that great progress is being made in our understanding of neurodegeneration and refined strategies are being developed to combat these diseases. A strong role for antibodies is becoming evident not only in fundamental research of misfolding, but also in early diagnosis and treatment. It remains to be seen however if antibody-based therapies will have as high impact as in other medical applications such as those developed for cancer therapy. The breakthroughs seen for the latter does give rise to strong hopes for equally effective antibody based therapies to treat neurodegenerative conditions.

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