Review

The contribution of biophysical and structural studies of protein self-assembly to the design of therapeutic strategies for amyloid diseases

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ABSTRACT

Many neurodegenerative disorders, including Alzheimer’s, Parkinson’s and the prion diseases, are characterized by a conformational conversion of normally soluble proteins or peptides into pathological species, by a process of misfolding and self-assembly that leads ultimately to the formation of amyloid fibrils. Recent studies support the idea that multiple intermediate species with a wide variety of degrees of neuronal toxicity are generated during such processes. The development of a high level of knowledge of the nature and structure of the pathogenic amyloid species would significantly enhance efforts to underline the molecular origins of these disorders and also to develop both accurate diagnoses and effective therapeutic interventions for these types of conditions. In this review, we discuss recent biophysical and structural information concerning different types of amyloid aggregates and the way in which such information can guide rational therapeutic approaches designed to target specific pathogenic events that occur during the development of these highly debilitating and increasingly common diseases.

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Contents

1. Introduction .................................................................................................................. 178
2. Amyloid formation and neurodegenerative disorders ................................................ 180
3. Neuronal toxicity of amyloid species ....................................................................... 180
4. Mechanisms of formation of amyloid aggregates .................................................... 181
5. Generic features of the structure of amyloid aggregates .......................................... 182
6. Amyloid formation pathways and therapeutic strategies ......................................... 184
References ....................................................................................................................... 187

1. Introduction

Amyloid formation refers to the process by which soluble, typically monomeric, peptides and proteins misfold and self-assemble into amyloid fibrils (Fig. 1). Amyloid fibrils are thread-like structures typically composed of several protofilaments with a common architecture, termed the cross-β structure (Eisenberg and Jucker, 2012; Fandrich and Dobson, 2002; Fitzpatrick et al., 2013; Jimenez et al., 1999; Sawaya et al., 2007; Sunde et al., 1997), which is largely independent of the amino acid sequence and native fold of the protein of which the fibrils are composed. This ability to form this structure has indeed been recognized to be a generic property of the polypeptide chain (Dobson, 1999, 2003).

For over a century, amyloid formation has been recognized as a hallmark of a number of medical disorders, particularly neurodegenerative diseases. Currently, approximately 50 distinct human diseases and disorders (Chiti and Dobson, 2017), some of them among the most common and debilitating medical conditions in the modern world, including Alzheimer’s (AD) and Parkinson’s disease (PD), have been linked with the accumulation of amyloid deposits, which are primarily composed of one specific type of peptide or protein depending on the particular disorder. Such conditions are collectively referred to as protein misfolding or amyloid diseases (Chiti and Dobson, 2006). Despite the well-established
of misfolded aggregated forms of the protein. The localization of protein with a gain of toxic function through the generation and accumulation of misfolded aggregated forms of the protein. The localization of protein inclusions in the damaged regions of patients suffering from amyloid diseases led initially to the hypothesis that the fibrillar species of which the inclusions are largely composed were the toxic agents responsible for pathogenicity in these diseases (Yankner et al., 1990). This may be the case for at least some systemic amyloidoses, in which large quantities of amyloid aggregates are deposited in vital organs (Chiti and Dobson, 2006). In other amyloid diseases, however, including most neurodegenerative disorders, there is a lack of a direct correlation between the extent of accumulation of protein inclusions and the severity of disease-associated symptoms, suggesting that other forms of aggregated proteins may be giving rise to neurodegeneration (Kalia et al., 2013; Ross and Poirier, 2005). Indeed, it is becoming evident from in vitro and in vivo experiments, and from analysis of biofluids from human patients, that some forms of oligomeric species could play an important role in these diseases (e.g., for proteins such as Aβ (Shankar et al., 2008; Townsend et al., 2006), tau (Lasagna-Reeves et al., 2012; Patterson et al., 2011), huntingtin (Nekooki-Machida et al., 2009) and α-synuclein (Cremades et al., 2012; Horrocks et al., 2016; Kalia et al., 2013; Tsigelnry et al., 2008; Winner et al., 2011). Such conclusions are also consistent with the hypothesis of generic effects of prefibrillar aggregates, even when the aggregates are not related with any known disease (Bucciantini et al., 2002; Dobson, 2003).

An increase in our detailed knowledge of the nature and structure of the pathogenic amyloid species and the details of their mechanisms of toxicity would be of very great value in the rational design of effective therapeutic strategies against these devastating diseases, for which there are still no effective treatments. In the last two decades, the pharmaceutical industry has devoted substantial resources into programs to develop drugs against AD and PD, primarily focused on the ‘amyloid cascade’ hypothesis. A variety of drug candidates with a diversity of molecular targets have been tested in clinical trials, some of them designed to inhibit the aggregation process, some to promote the removal of amyloid deposits, and some to modulate the transport of the amyloid protein precursors between the central nervous system and the peripheral circulation (Folch et al., 2016). However, almost none of the drug candidates tested so far has shown any significant efficacy or succeeded in clinical trials (see for example (Folch et al., 2016)).

Immunotherapy using antibodies against a specific amyloidogenic protein, also referred to as immunomodulation through passive immunization, has been suggested as a potential therapeutic treatment for amyloid diseases in recent years (see for example (Valera et al., 2016)), particularly since the recognition that toxic amyloid aggregates can be involved in the spread and propagation of the neurodegenerative progression of disease by means of a ‘prion-like’ mechanism (i.e., through the spatial propagation of amyloid assemblies from cell to cell coupled with seeding and secondary processes) (Brundin et al., 2010; Danzer et al., 2009; Jucker and Walker, 2013; Luk et al., 2012). In the case of extracellular peptides and proteins such as the Aβ peptide, involved in AD, it has been suggested that amyloid aggregates can be taken up by adjacent neuronal cells, leading to pathological propagation, while for intracellular proteins such as α-synuclein (αS), linked to PD, the initially generated intracellular aggregates are assumed to enter the extracellular space and then to be taken up by neighbouring cells (Holmes and Diamond, 2012; Luk et al., 2012; Peelherts et al., 2015; Volpicelli-Daley et al., 2011). Thus immunotherapy aiming to reduce the level of extracellular toxic amyloid aggregates, or preventing them from entering cells and spreading into new regions, could be a promising therapeutic strategy to prevent the progression of pathology (Mandler et al., 2015; Sankaranarayanan et al., 2015; Tran et al., 2014; Valera and Masliah, 2016). Indeed, preclinical studies have shown that passive immunization with this aim reduces cerebral amyloid load, improves cognition, and mitigates neurodegeneration in mouse models (Bard et al., 2000; Kalia et al., 2013; Oddo et al., 2004; Wilcock et al., 2004).

One current limitation of such immunotherapy approaches is that many of the antibodies that have been developed and are under investigation do not discriminate between different types of protein aggregates and are typically raised against, or preferentially bind to, the monomeric form of the protein (Goure et al., 2014). As this is typically the biologically active form of the protein, however, the binding to an antibody can potentially perturb very significantly its biological function. In addition, viable dosages of these antibodies may be saturated by the high levels of the monomeric form of the protein relative to the pathogenic forms present in vivo. Indeed, preferential binding to the monomeric form of the protein may be a significant contributor to the failure of clinical trials to show improvement of disease symptoms (Doody et al., 2014; Goure et al., 2014). Taken together, these studies have suggested that non-specific targeting, or the targeting of monomers, is unlikely to be effective in a clinical setting, and much efforts have been devoted for the development of immunotherapies that can selectively target the most highly toxic amyloid species (see for example (Folch et al., 2016)). Antibodies that target a region of the peptide sequence may, however, prove to have low efficacy, as they may cross-react with multiple forms of the amyloidogenic polypeptide...
instead of being specific to the most highly toxic species. Instead, conformational or structurally specific antibodies (De Genst et al., 2014; Kayed et al., 2007, 2003; Perchiacca et al., 2012) could be the key to targeting specific toxic aggregated forms provided it becomes possible to develop structurally specific antibodies that are able to differentiate between aggregate polymorphs or strains of the same protein; in this way it may be possible to target selectively different pathological features that occur during the development of a particular amyloid disease.

### 2. Amyloid formation and neurodegenerative disorders

The amyloid cascade hypothesis was put forward in the 1990s and suggested that the formation and accumulation of fibrillar amyloid aggregates is the primary origin of the pathogenesis of AD (Hardy and Higgins, 1992; Yankner et al., 1990). This hypothesis emerged from the recognition that amyloid fibrillar forms of the Aβ peptide are a common feature of AD patients (Glenner and Wong, 1984; Masters et al., 1985). Since then, the aggregation of more than 30 peptides and proteins have been linked with other amyloid diseases (in some cases the same protein is involved in multiple diseases), with a significant number involved in brain diseases, notably neurodegenerative disorders (Chiti and Dobson, 2006; Knowles et al., 2014). Indeed, it is well established that over-production or imbalance in the generation/degradation of a specific amyloidogenic peptide or protein plays a role in neurodegeneration and the development of disease (Mucke, 2009).

Additional support for this hypothesis came later from genetics, where single point mutations in the various genes that encode specific amyloidogenic proteins could be linked with the onset of familial forms of disease, particularly in the case of AD and PD (Bekris et al., 2010; Klein and Westenberger, 2012). In most of these familial cases, fortunately relatively rare, there is early onset of the disease but the pathophysiology is essentially identical to that of the sporadic, late-onset forms (Bekris et al., 2010; Goate et al., 1991; Klein and Westenberger, 2012). A significant number of mutations have now been observed in the genes that encode the amyloid precursor protein (APP) and αS that are associated with early-onset familial cases of AD and PD, respectively, and most of them have been related to an increase in either the levels of the amyloidogenic protein or in its aggregation propensity (Irvine et al., 2008).

In the case of αS, in addition to familial mutations, duplication and triplication of the αS gene can occur, resulting in increased levels of the wild type protein that are sufficient to cause autosomal dominant early-onset forms of PD (Klein and Westenberger, 2012). Although these familial cases account for only a small fraction of PD in the general population, the Lewy bodies and Lewy neurites observed both in familial and idiopathic PD stain strongly for αS (Galvin et al., 1999; Spillantini et al., 1998; Spillantini et al., 1997). In addition, a genome-wide association study has shown that individuals with certain polymorphisms of the gene that encodes for αS have a higher risk of developing sporadic PD (Seidel et al., 2010), and some of these polymorphisms have been associated with higher expression levels of αS in neurons (Maragano et al., 2006). In an analogous way, internal chromosome 21 duplications or triplication of chromosome 21 in Down syndrome that results in over-expression of the APP has also been linked with brain pathology and higher risks of early-onset AD (Head et al., 2012). Conversely, partial trisomy of chromosome 21 that does not result in extra APP gene does not lead to AD (Sleegers et al., 2006). It is clear, therefore, in both AD and PD, that an increase in quantities of the amyloidogenic protein results in an increased risk of the onset of disease.

Although it is widely known and well established that aggregation of a particular protein is the hallmark of each of these types of disease (amyloid pathology is observed in all cases), and that there is a direct genetic link between amyloidogenic proteins and the development of disease, the relationship between amyloid deposition with cognitive decline is a subject of intense debate in the field. Indeed, the amount and location of the amyloid inclusions do not always correlate with neuronal loss (Delacourte et al., 1999; Gomez-Isla et al., 1996), and the recent clinical failures of potential AD drugs targeting the Aβ peptide have intensified scrutiny of the amyloid cascade hypothesis. As a result, alternative hypotheses have been suggested for the onset and progression of AD and PD, such as the mitochondrial cascade hypothesis (see review (Swerdlow et al., 2014)) or the metabolism hypothesis (Hoyer, 1991; Hoyer et al., 1988). None of these ideas, however, succeeds in reconciling individually all of the experimental and clinical aspects of these types of disease.

An alternative to the amyloid cascade hypothesis that has currently gained significant momentum is one in which low molecular weight oligomers generated during the formation of the amyloid fibrils represent key neurotoxic agents that cause neurodegeneration (Chiti and Dobson, 2006; Dobson, 2003; Walsh and Selkoe, 2007). Oligomeric forms of amyloid aggregates have been detected in the brains and tissues of patients suffering from neurodegenerative disorders and growing experimental evidence suggests that certain oligomeric forms of amyloidogenic proteins are inherently more damaging than their fibrillar counterparts, and are believed to be the primary origins of the toxicity associated with amyloid diseases (Arrasate et al., 2004; Bucciantini et al., 2002; Haass and Selkoe, 2007; Winner et al., 2011). The nature of their particular toxic activity has been long hypothesized to arise from a set of unique structural features characteristic of these species that are absent in the monomeric and in the fibrillar amyloid forms (Evangelisti et al., 2016; Kayed et al., 2003), in combination with their diffusive capabilities as a result of their relatively small size, which could lead them to mediate toxic effects at regions distant from the location of the amyloid plaques. Important evidence for a toxic role of amyloid oligomers comes from studies where some single point mutations in the amyloidogenic polypeptides, some of them related with familial, early onset forms of the disease, have been shown to favour the accumulation of oligomeric rather than fibrillar aggregates and to show a more toxic behaviour in vivo (Goate et al., 1991; Winner et al., 2011). In addition, oligomeric forms of Aβ and αS have been shown to promote amyloid aggregation and deposition (Cohen et al., 2013; Walker and Jucker, 2015) and have been suggested to propagate between cells in a prion-like manner (Ye et al., 2015). Although the identity of the most pathological forms of amyloid assemblies is still a subject of intense debate in the field, particularly in the recent years (Luk et al., 2012; Peelaerts et al., 2015; Prusiner et al., 2015; Woerman et al., 2015), the causal role of amyloidogenic proteins in neurodegenerative diseases is widely considered to involve soluble amyloid oligomers.

### 3. Neuronal toxicity of amyloid species

Aberrant folding and aggregation of certain amyloidogenic proteins has been suggested to be the initial trigger of amyloid diseases that is then followed by other events, such as calcium and metal ion imbalance, oxidative stress, and the overload of chaperone and ubiquitin proteasome systems (Irvine et al., 2008; Ross and Pickart, 2004). The generic ability of proteins to undergo similar conformational transitions resulting in the acquisition of a characteristic β-sheet rich structure, albeit displaying differences in size and overall morphology, points to common pathways of aggregation with common mechanisms of toxicity (Bucciantini et al., 2002; Dobson, 1999).

Three distinct pools of protein species can be distinguished once the self-assemble process has been triggered: monomers, soluble oligomers, and insoluble fibrillar species (Fig. 1). Each of these pools encompasses an array of individual species, particularly in the case of soluble oligomers, which globally can show very high heterogeneity in terms of size and structure. While neither monomeric Aβ nor αS has been reported so far to possess any direct cellular toxicity under physiologically relevant concentrations, amyloid aggregates generated by these two polypeptides have been shown to exhibit substantial neurotoxicity (Knowles et al., 2014). A significant number of studies have proposed that amyloid plaques and insoluble fibrillar aggregates of both Aβ and...
αS exhibit relatively low in vitro toxicity and that their formation could be actually beneficial as an in vivo mechanism for removal of the more toxic soluble oligomers (Baglioni et al., 2006; Cheng et al., 2007; Dobson, 2003; Treusch et al., 2009). In contrast, since the first suggestion that soluble oligomeric species rather than fibrillar plaques could trigger neurotoxicity, many studies have shown that soluble oligomers are the most toxic forms of amyloidogenic proteins, able to induce neurodegenerative processes (Benilova et al., 2012; Bucchianni et al., 2002; Ferreira and Klein, 2011; Hayden and Teplov, 2013; Sakono and Zako, 2010; Winner et al., 2011). In addition, biochemical studies have demonstrated a strong correlation between the levels of soluble amyloid oligomers in the brains of patients with AD and PD and the degree of cognitive impairment (Ferreira et al., 2014; Winner et al., 2011). Additionally, blocking Aβ oligomerization but not fibril formation has been shown to protect cells from toxicity (De Felice et al., 2004; Habchi et al., 2016b) and similarly mutants of αS that promote oligomer but not fibril formation have been reported to induce the most severe dopaminergic neuronal loss in the substantia nigra of animal models (Winner et al., 2011). In fact, amyloid fibrils have been proposed to represent the end stage of protein aggregation, acting as scavengers of misfolded soluble oligomers that are more toxic and also resistant against degradation by proteasomes (Benilova et al., 2012).

Although the detailed mechanisms by which soluble self-assembled oligomers could trigger toxicity remains to be established, membrane perturbation has been proposed as a general primary step to neurodegeneration by these aggregates. However, other cytotoxic effects of amyloid oligomers have been also proposed, including induction of oxidative stress (De Felice et al., 2007; Deas et al., 2016), mitochondrial dysfunction (Devi et al., 2008; Nakamura, 2013), impairment of the proteasome system (Xilouri et al., 2013) and blocking of trafficking in the ER-Golgi (Cooper et al., 2006). It has been proposed generally that oligomers are able to interact directly with and disrupt membranes, although there is much to be established on the details of the mechanism of membrane disruption (Stefani and Dobson, 2003), some groups pointing towards a pore-like mechanism (Kayed and Lasagna-Reeves, 2013; Serra-Batiste et al., 2016; Yoshiike et al., 2007) and others to membrane destabilization without the need for the formation of specific pores in the membrane (Brender et al., 2012; Flagmeier et al., 2017; Stockl et al., 2013; Walsh et al., 2014). In addition, other studies have shown that some amyloid oligomers, particularly those from Aβ, might exert their toxic effects through their interaction with specific membrane receptors (Lauren et al., 2009; Li et al., 2011; Morkuniene et al., 2015). Regardless of the specific mechanism of interaction, it is well accepted that membrane perturbation by toxic soluble oligomers can result in dysregulation of signal transduction, changes in ion homeostasis in the cell, increases in intracellular calcium levels and induction of oxidative stress, likely to be a result of the entry of extracellular calcium and redox-active metal ions into the cells due to disruption of membrane permeability (Angelova et al., 2016; Arispe et al., 1993; Deas et al., 2016; Pedersen et al., 2016; Quist et al., 2005; Stefani and Dobson, 2003). Recent studies indicate that amyloid aggregates could also induce inflammation through a receptor-mediated mechanism or by direct interaction with the membrane (Sengupta et al., 2016). Inflammation has long been a known component of AD and PD, and, indeed, inflammation is one of the earliest signs of AD, beginning prior to the formation of visible Aβ plaques. It is, however, still unresolved whether inflammation precedes aggregate formation or if aggregation formation induces an inflammation response (Minter et al., 2016).

There is, therefore, continuous discussion as to whether the fibrillar species and amyloid plaques are more damaging than soluble oligomeric species (Goure et al., 2014; Peelaerts et al., 2015) and whether or not the former represent a sink or a source of soluble oligomeric species. Indeed, recent studies have shown that oligomeric species can be generated via fibril disaggregation processes (Cremades et al., 2012; Kim et al., 2008; Martins et al., 2008) and by secondary nucleation mechanisms caused by amyloid fibrils where the fibrillar surface can catalyze the formation of oligomeric species (Cohen et al., 2013; Jeong et al., 2013). These oligomer formation processes are likely to result in a significant local concentration of toxic oligomers around the plaques and, given their soluble and diffusive nature, they could easily reach distant regions and give rise to cellular damage; indeed, these conclusions are in agreement with in vivo studies where a halo of soluble oligomers surrounding amyloid plaques has been reported (Koffie et al., 2009). In addition, recent studies suggest a correlation between levels of soluble amyloid oligomers in cerebrospinal fluid and cognitive deficits in human patients with AD (Savage et al., 2014) and PD (Horrocks et al., 2016; Kalia et al., 2013), consistent with the view that soluble amyloid oligomers interfere with synaptic function and contribute to cognitive dysfunction in these types of disease. By contrast, other studies have suggested that the fibrillar species themselves could be highly toxic (Luk et al., 2012; Peelaerts et al., 2015; Prusiner et al., 2015; Woerman et al., 2015) and may be responsible for the spreading of disease by seeding the conversion of functional soluble monomeric protein into fibrillar structures in the cytoplasm of recipient cells (Desplats et al., 2009; Hansen et al., 2011; Luk et al., 2012; Peelaerts et al., 2015; Pieri et al., 2016).

Taken together, therefore, these studies support the conclusion that multiple protein species generated during protein amyloid aggregation, rather than a single, discrete toxic species, exhibit neuronal toxicity probably as a result of the triggering of different neuronal activities and with the potential to induce different levels of toxicity, perhaps through different types of interactions with cellular components, depending on the details of their structure and the nature of the cells in which they are formed or to which they are exposed (Campioni et al., 2010; Cremades et al., 2012; Peelaerts et al., 2015).

4. Mechanisms of formation of amyloid aggregates

The mechanisms by which proteins convert from their functional soluble forms into the amyloid state have been shown to be highly complex and the process is known to depend on both the intrinsic characteristics of the protein and the environmental conditions under which aggregation occurs. Early analysis of the kinetics of formation of amyloid fibrils demonstrated that the overall process follows a nucleation-polymerization model (Jarrett and Lansbury, 1992), where soluble species undergo a nucleation step that results in the formation of oligomeric species that are then able to grow through further monomer addition, eventually generating mature fibrils (Fig. 1). The growth profile of such a process (Fig. 2, left) is characteristically sigmoidal, reflecting the greater ease of addition of monomers onto existing aggregates compared to the de novo formation of new oligomers directly from monomers. The overall reaction rate therefore accelerates when significant numbers of aggregates are present in solution, resulting in an initial lag phase followed by a rapid growth phase before a plateau region is reached when the monomer concentration becomes depleted (Cohen et al., 2011b; Knowles et al., 2014).

There are generally two processes that generate new aggregates: primary nucleation processes where new aggregates form at a rate dependent only on the concentration of monomeric species, and secondary processes where new aggregates are formed at a rate dependent on the concentration of existing fibrils (Cohen et al., 2013). The secondary processes that generate new aggregates can in turn be subdivided into monomer-independent processes, such as fragmentation, and monomer-dependent processes, such as secondary nucleation, where the surfaces of existing fibrils catalyze the nucleation of new aggregates from the monomeric state (Cohen et al., 2011b; Knowles et al., 2009). Importantly, it has been observed that in some cases, surface catalyzed nucleation can be a dominant factor in the generation of toxic oligomers (Cohen et al., 2013).

Amyloid formation can also be seeded by the addition of pre-formed fibrils, a phenomenon analogous to that observed in crystalization, in which the primary nucleation step is essentially by-passed. In the
presence of a significant number of seeds and under conditions where predominant secondary processes are absent, the aggregation profile is then expected to follow a single exponential function (Fig. 2, right) (Cohen et al., 2011a), as a consequence of the much slower rate (higher energy barrier) of primary nucleation compared to the rate of addition of monomers onto an existing fibril. An interesting property of the fibril seeding process is that the structural features of the seeds are generally reproduced in the growing fibrils. This phenomenon is sometimes called templating (Serio et al., 2000), and once a template with a cross-β structure (typically pre-formed fibrils) is introduced into a solution containing monomeric protein, molecules of monomers bind to the fibril ends and adopt the cross-β structure, becoming the next attachment/templating surfaces of the fibril, again by analogy with the processes that takes place in crystallization.

This type of seeding and templating process has been proposed to be the basis by which the misfolded prion protein acts as an infectious agent to propagate prion diseases (Aguzzi, 2009; Soto, 2012; Westmark and Westmark, 2010) and is also likely to be an important factor in the propagation of other amyloidogenic diseases (Brundin et al., 2010; Jucker and Walker, 2011; Walker and Jucker, 2015). Although the role of amyloid spreading and propagation in disease pathogenesis was first recognized to be important for the neurodegenerative diseases caused by the misfolding of prions, increasing evidence suggests that this self-propagating activity is present in most, or all, amyloid proteins and peptides involved in other amyloid diseases (Walker and Jucker, 2015) and is the ultimate consequence of the templating properties of the cross-β structure. For example, it has been recently shown for a number of amyloid polypeptides, including Aβ and αS, that amyloid propagation with high structural and pathological fidelity can be triggered from exogenous sources of amyloid aggregates (Lundmark et al., 2002; Peelaerts et al., 2015; Stohr et al., 2012; Volpicelli-Daley et al., 2011), sharing analogies to the strain-specific infectivity long observed for the prion proteins (Prusiner, 1991). The existence of functional prions and amyloid structures at physiological conditions challenges the generality of this idea, as they seem to exploit the self-assembling and templating properties of the cross-β structure but lack pathogenicity. In these cases, however, a robust cellular control of the aggregation mechanisms that could avoid the generation of pathogenic amyloid species is likely to exist (Maji et al., 2009). Moreover different types of behaviour will be observable under different conditions. Thus, in a situation where the rate of secondary nucleation is very slow, other microscopic processes (e.g. primary nucleation and elongation) can become dominant, and the mechanism of rapid proliferation through secondary nucleation will become negligible. Under these circumstances, that may be common for functional amyloid species, the aggregation process is much more readily controlled.

In conclusion, primary and secondary aggregation pathways as well as seeding mechanisms are likely to play important roles in disease through the de novo formation of aggregates, the incorporation of functional proteins into misfolded states and the multiplication and proliferation of toxic aggregates that can not only damage or kill the cells in which they form, but also invade neighbouring healthy cells and accelerate the conversion of soluble protein molecules into fibrils.

5. Generic features of the structure of amyloid aggregates

Amyloid fibrils are referred to as protein assemblies with a characteristic cross-β architecture, in which β-strands are formed and aligned perpendicular to the fibril axis, generating inter-molecular β-sheets that run the length of the aggregate. Amyloid fibrils are typically long unbranched thread-like structures, just a few nanometers in diameter, and composed of several protofilaments with the classic cross-β structure that twist around each other (Chiti and Dobson, 2006, 2017; Eisenberg and Jucker, 2012; Fitzpatrick et al., 2013; Jimenez et al., 1999, 2002; Sawaya et al., 2007; Sunde et al., 1997) (Fig. 3A). The origins of the cross-β architecture can be attributed to the nature of the intra- and intermolecular interactions within the β-sheets, which are dominated by hydrogen bonds between the main-chain atoms of the polypeptide chain that are common to all peptide and protein molecules (Fandrich and Dobson, 2002; Knowles et al., 2007). A consequence of this particular structure is that the spacing between strands forming the β-sheets along the fibril axis is determined by the inter-main chain hydrogen bonding constraints and is, therefore, independent of the amino acid sequence of the polypeptide, and has a value close to 4.7 Å. By contrast, the inter-sheet spacing (i.e., the spacing between β-sheets in the direction perpendicular to the fibril axis) is variable (typically 9–11 Å) and highly dependent on the nature of the side chains (Fandrich and Dobson, 2002; Knowles et al., 2014). The structural variations between amyloid fibrils generated from different polypeptide chains, therefore, arise primarily from the manner in which the various side chains are incorporated into the cross-β structure (Fitzpatrick et al., 2013; Jimenez et al., 1999; Serpell and Smith, 2000; Serpell et al., 2000, 1995; Zhang et al., 2009) (Fig. 3A).

The details of the characteristic structure of amyloid fibrils are also shedding light into the details of the mechanisms by which seeding and templating at the fibril ends occur. While the architecture of the fibrils is not strongly dependent on polypeptide sequence, since the main interactions that stabilize the fibril core consist on an array of
interbackbone hydrogen bonds (Dobson, 1999; Eisenberg and Jucker, 2012; Fitzpatrick et al., 2013; Knowles et al., 2007), self-complementary interdigitation of the side chains between polypeptides within the same β-sheet and between β-sheets have been proposed to be important to create a tightly packed hydrophobic fibrillar core (Eisenberg and Jucker, 2012; Fitzpatrick et al., 2013; Luhrs et al., 2005). This packed interdigitation between side chains explains both why templating between identical polypeptide chains, rather than between differing sequences, and the parallel in-register topology are the optimal configurations in the amyloid fibrils where structures have been defined at present (Fitzpatrick et al., 2013; Heise et al., 2005; Petkova et al., 2006; Tuttle et al., 2016). In addition, in certain fibrillar structures, a slight shift between β-sheets has been observed, which leads to incompletely paired β-strands at the fibril ends, with exposed hydrophobic residues and unsatisfied hydrogen bonds; such features are likely to encourage the recruitment of additional polypeptide chains, which, by adopting the cross-β structure, could stabilize further the growing fibril ends, leading to increasing extension of the fibrillar structure (Fitzpatrick et al., 2013; Luhrs et al., 2005).

Another interesting feature of the cross-β structure is the multiplicity of distinct morphologies of amyloid fibrils that a particular protein can adopt in response to mutations in its sequence (Bolognesi et al., 2014; Hess et al., 2007) or upon different aggregating conditions (Gorski et al., 2001; Petkova et al., 2005), and even observed to coexist under the same conditions (Goldsbury et al., 2005; Heise et al., 2005; Kad et al., 2003). The hierarchical organization of amyloid fibrils, from the configuration and arrangement of the β-strands into β-sheets, to the packing of the β-sheets into protofilaments and the assembly of protofilaments into filaments and ultimately mature fibrils, gives rise to multiple polymorphisms of amyloid assemblies that may occur at the different stages of the self-assembly process, resulting in variations both at the molecular and ultrastructural level of the fibril (Chiti and Dobson, 2006, 2017; Eisenberg and Jucker, 2012). Indeed, different segments of identical polypeptides may be involved in the core structures, resulting in fibrils with different conformational features (Colletier et al., 2011; Lewandowski et al., 2011; Luhrs et al., 2005; Petkova et al., 2002; Wasmer et al., 2008), and variations in the number of constituent protofilaments and in the manner in which they interact between each other can lead to multiple ultrastructural polymorphisms (Bauer et al., 1995; Fitzpatrick et al., 2013; Jimenez et al., 2002; Schmidt et al., 2009; Zhang et al., 2009).

While significant advances have been made towards understanding the structures of fibrillar aggregates, of particular significance in terms of pathogenicity is the structural information that is beginning to emerge for the oligomeric species that are frequently observed to accumulate during the process of formation of fibrils (Bemporad and Chiti, 2012; Campioni et al., 2010; Chen et al., 2015; Cremaides et al., 2017; Kayed et al., 2003; Serra-Batiste et al., 2016). At a fundamental level, the rapid elongation rate of small aggregates in the presence of monomers makes the study of transient oligomeric species generated during the formation of fibrils particularly challenging. The ability to produce and isolate trapped oligomeric forms has, however, opened up the possibility of gaining insights into the nature and structure of these species (Campioni et al., 2010; Celej et al., 2012; Chen et al., 2015; Ding et al., 2002; Lorenzen et al., 2014b; Paslawski et al., 2014; Serra-Batiste et al., 2016; van Rooijen et al., 2009; Volles et al., 2001).

Particular efforts have been focused on identifying the nature and structure of oligomers of αS. A variety of procedures have been used to trap αS oligomeric forms; some involving a change in the physicochemical conditions under which aggregation occurs, others involving the covalent modification of the protein or the addition of chemical compounds able to interact with certain types of oligomers and inhibit their elongation and conversion into fibrils (Cremaides et al., 2017). The evident variability in the type of αS oligomeric species generated by the different methods is highly likely to reflect the presence of multiple aggregation processes, although the data collected so far on the various αS oligomeric species suggests that it is likely that only a relatively small number of preferred self-assembly pathways exists (Cremaides et al., 2017).

We, and others, have used lyophilization as a way of trapping and enriching samples containing one type of toxic oligomeric species generated by αS (Celej et al., 2012; Chen et al., 2015; Galea and Celej, 2014; Lorenzen et al., 2014b; Paslawski et al., 2014; van Rooijen et al., 2009), which has allowed us to obtain detailed structural and biophysical information on the nature of such species (Chen et al., 2015). The enriched samples are composed of oligomers with a range of sizes, with an average of approximately 25–30 protein molecules, although in some cases the size distribution of the
enriched oligomers indicate that the sample contains 10–90 mers (Chen et al., 2015; Lorenzen et al., 2014b; Zijlstra et al., 2012). These oligomers have been found to have remarkable stability (Chen et al., 2015; Paslawski et al., 2014) and to be able to associate with lipid bilayers and induce membrane permeability (Chen et al., 2015; Giehm et al., 2011; Lorenzen et al., 2014a). The β-sheet content of these oligomers is intermediate between that of the intrinsically disordered monomers and that of the fibril structure.

The core structure of the oligomers appear to involve similar residues to that of the fibril, although the latter involves a larger number of residues and has a parallel β-sheet arrangement (Celej et al., 2012; Chen et al., 2015; Gallea and Celej, 2014), in contrast to the primarily anti-parallel β-sheet conformation of this type of oligomer (Celej et al., 2012; Chen et al., 2015; Gallea and Celej, 2014; Lorenzen et al., 2014b; Paslawski et al., 2014). Interestingly, all of the information accumulated so far on these αS oligomers indicates that they have a remarkably high degree of similarity, in terms of physico-chemical, structural, and toxic properties, with oligomeric species formed by αS under different conditions and indeed other amyloidogenic proteins and peptides (Bemporad and Chiti, 2012; Chen et al., 2015; Danzer et al., 2007; Gallea and Celej, 2014; Lorenzen et al., 2014b; Paslawski et al., 2014; van Rooijen et al., 2009; Volles et al., 2001; Zijlstra et al., 2012). Indeed, the difference in the organization of the β-sheets, from a dominance of parallel β-sheet structure in the fibrillar form to antiparallel β-sheet structure in stable oligomeric species, has also been reported for several other amyloidogenic peptides and proteins such as the Aβ-peptide (Cerf et al., 2009), lysozyme (Zou et al., 2013), a prion-related peptide (Natalello et al., 2008), and β2-microglobulin (Fabian et al., 2008).

Importantly, we found that the stable αS oligomers are toxic when exposed to neuronal cells (Angelova et al., 2016; Chen et al., 2015; Creemades et al., 2012; Deas et al., 2016) and reproduce most of the pathophysiological features of PD in model neurons (Angelova et al., 2016; Deas et al., 2016; Devine et al., 2011). Although they are in principle able to elongate and act as seeds for the recruitment of monomeric protein molecules, however, these oligomers appear to do so only very slowly under physiologically-relevant conditions; indeed, the elongation rate for these oligomers under such conditions is at least three orders of magnitude slower than that of the parallel β-sheet fibrillar structures (Chen et al., 2015), a feature attributable at least in part to the anti-parallel β-sheet structure that is predominant in these oligomers (Chen et al., 2015; Qiang et al., 2012), and would need, therefore, to undergo extensive structural reorganization in order to form stable fibrils.

The strategy of using a wide variety of biophysical methods to study these specific αS oligomers has enabled us to reduce their heterogeneity to such an extent that it has been possible to begin to define their overall structural properties and molecular architectures. We found that all the oligomeric species present in the sample belong to the same structural family, differing primarily only in their size and their β-sheet content; indeed we observed also that the β-sheet content in fact depends on the size (the larger the oligomer, the greater the β-sheet content) (Chen et al., 2015). Importantly, we were able to use cryo-EM image reconstruction techniques to generate a low-resolution structural model, revealing a cylindrical architecture with a central cavity. This model has remarkable similarities to those of mature amyloid fibrils formed by a variety of proteins, despite their differences in morphology and amino acid sequences (Chen et al., 2015; Chiti and Dobson, 2006). The similarities in the architecture of the fibrillar and the oligomeric forms, despite the differences in the organization of their β-sheet structure, suggest, therefore, that similar types of interactions to those that stabilize amyloid fibrils are likely to be responsible for the initial acquisition of cross-β structure in the oligomeric species. Moreover, oligomeric species, formed during fibril formation observed by TEM and AFM, with remarkable similarities to the 3D cryo-EM model of antiparallel β-sheet oligomers have been reported for αS and other amyloidogenic polypeptides (Lasnuel et al., 2002; Quist et al., 2005).

These species, like the antiparallel β-sheet oligomers, are also capable of binding the amyloid oligomer-specific A11 antibody and being highly cytotoxic (Kayed et al., 2003), suggesting that the transient oligomeric forms generated during fibril formation, which are likely to have an amyloid core composed primarily of parallel β-sheet, and the kinetically-trapped antiparallel β-sheet oligomers could be analogous in their global structure and represent different types of cross-β oligomeric precursors. Interestingly, the A11 and other related antibodies have also been widely used to monitor the presence of oligomers in AD brains, and the results indicate that such oligomers are likely to exist in vivo and to be related to pathogenicity (Hillen et al., 2010; Kayed et al., 2007, 2003; Lacor et al., 2004; Lasagna-Reeves et al., 2012; Noguchi et al., 2009). Indeed, some of the characteristics reported for the toxic oligomers of αS found in vivo are very similar to those of the β-sheet rich oligomers shown to form in vitro, both during the fibril formation reaction and as a result of the lyophilization process (Emmanouilidou et al., 2010; Feng et al., 2010; Pountney et al., 2004; Roberts et al., 2015). All these findings suggest that the underlying architecture reported for the αS oligomers can be adopted by different peptides and proteins, regardless of their amino acid composition and sequence (Chen et al., 2015), as proposed for the fibrillar structures (Chiti and Dobson, 2006; Dobson, 2003; Eisenberg and Jucker, 2012), and are likely to play an important role in disease.

6. Amyloid formation pathways and therapeutic strategies

The lack of efficacy of Aβ and αS immunotherapies in clinical studies has been associated with a range of different factors, including the extent of disease progression in those patients involved in the trials. Of additional importance, however, is undoubtedly the lack of selectivity for the most highly toxic amyloid species involved in the initiation and progression of disease (Folch et al., 2016; Karan and De Strooper, 2016; Sengupta et al., 2016); indeed all the antibodies currently under development bind to either monomeric or fibrillar species or to both, and there is likely to be in general a large excess of monomeric or fibrillar species in the brains of AD or PD sufferers relative to soluble amyloid oligomers. Studies have demonstrated, however, that antibodies with selective affinity for soluble Aβ oligomers can block the toxicity associated with these species in cell cultures (Lambert et al., 2007; Shughrue et al., 2010) and reduce memory deficits in transgenic AD mouse models (Hillen et al., 2010), supporting the concept that antibodies with selective affinity for specific types of amyloid oligomers may be a more effective therapeutic strategy to block neurodegeneration.

Characterization of the oligomeric species generated during the formation of amyloid fibrils is, however, very challenging, particularly as a consequence, at any time of the reaction, of their often low populations relative to monomeric of fibrillar species and their highly heterogeneous nature. In order to overcome these difficulties, a number of techniques have been developed, including single-molecule methods that enable the direct observation and characterization of individual molecular species populated during the aggregation reaction (Creemades et al., 2012; Ding et al., 2002; Orte et al., 2008; Pitschke et al., 1998). Based on these and related studies it has become evident that multiple types of oligomeric species are formed during the early stages of the self-assembly process that leads to amyloid fibrils, and studies are beginning to characterize their nature (Creemades et al., 2012; Iljina et al., 2016). In particular, single-molecule fluorescence methods in which intermolecular Förster resonance energy transfer (FRET) effects can be monitored have been shown to be extremely valuable for identifying specific types of oligomeric species and following their formation and evolution during the early stages of protein aggregation (Creemades et al., 2012).

Investigations of the primary nucleation process of fibril formation by αS using this methodology have revealed the existence of two important and distinct groups of oligomers. Specifically it has been found...
that αS monomers self-assemble initially into relatively disordered and benign oligomers, and that they subsequently convert into much more stable oligomers, characterized by extensive β-sheet structure and a highly toxic nature. These oligomers ultimately convert into amyloid fibrils by slow rearrangement (Fig. 4A) (Cremades et al., 2012; Iljina et al., 2016) that is likely to occur through a process of sequential folding upon self-assembly (Cremades et al., 2017). A similar mechanism has been proposed for the aggregation of αS in the presence of phospholipid vesicles, with the rates of oligomeric conversion being comparable to those found for the aggregation of the protein without lipid membranes (Galvagnion et al., 2015).

The conversion from disordered to amyloid-like β-sheet oligomers appears to be a key step in the self-assembly process that leads not only to fibril formation but also to toxicity and resistance to degradation by the cellular proteostasis machinery (Cremades et al., 2012) (Fig. 4B). This conversion process is also remarkably slow indicating that the two structurally distinct types of oligomers are likely to co-exist, particularly at the early stages of the self-assembly process, suggesting that there will be a significant period of time for the cellular protective machinery to operate, for example by sequestering certain types of oligomers (Arosio et al., 2016). This period of time (Fig. 4B) also suggests that therapeutic intervention may be viable prior to the onset of cellular damage (Cremades et al., 2012). For such purposes, it will be of the utmost importance to identify the differences between the specific physicochemical and structural features of the different aggregates in order to target specifically the initially formed disordered oligomers and prevent their conversion into toxic amyloid oligomers (Fig. 4B). Indeed, this system could form the basis for the development of the specific features that give rise to toxicity.

The close similarity between the global architecture of the stable toxic αS oligomers, which we have characterized in some detail (Chen et al., 2015), and that found to be characteristic of at least a number of disease-associated fibrils, suggests that similar types of interactions (notably an array of inter-backbone hydrogen bonds linking the β-strands as in the fibrillar structures (Knowles et al., 2007, 2014)) is likely to stabilize both types of species. Consequently, a high degree of heterogeneity of β-sheet oligomers with the same type of core architecture but with different numbers and lengths of β-strands and different types of β-sheet arrangements, and indeed permutations of interstrand hydrogen bonding interactions, are expected. Indeed, such species could be the precursors of structurally distinct but energetically similar fibril polymorphs that have been observed to trigger marked differences in cellular vulnerability and pathology (Prusiner et al., 2015; Winner et al., 2011; Woerman et al., 2015).

Some of these β-sheet-rich oligomers with a significant degree of heterogeneity in their β-sheet content can accumulate once they are formed as a consequence of their extremely slow rates of assembly, disassembly and reorganization of cross-β structure (Cremades et al., 2012). Thus, an array of β-sheet-rich oligomers could accumulate during the process of amyloid self-assembly, which in turn could have marked effects on the type and extent of toxic effects they induce in cells. Indeed, we have proposed that differences in the arrangement of the β-strands between certain β-sheet-rich oligomers and fibrils could be a major reason for the very large differences between their relative...
ability to elongate and to propagate (Chen et al., 2015); whereas the parallel \(\beta\)-sheet arrangement of the amyloid structure is readily able to increase in length, the antiparallel \(\beta\)-sheet configuration appears to have a much lower tendency to grow by further addition of monomers (Chen et al., 2015; Petkova et al., 2004; Qiang et al., 2012). In this way, a rearrangement of the \(\beta\)-strands from an antiparallel to a parallel configuration would then be required for the efficient elongation of oligomers, which have acquired predominantly an antiparallel \(\beta\)-sheet amyloid configuration during the self-assembly process, in order to generate the fibrillar amyloid architecture under physiological conditions (Chen et al., 2015; Petkova et al., 2004; Qiang et al., 2012). The exceptional kinetic stability of such species, together with their high thermodynamic stability and resistance to proteolysis (Chen et al., 2015), and coupled with their highly toxic nature (Chen et al., 2015; Lorenzen et al., 2014b; Volles et al., 2001), make these antiparallel \(\beta\)-sheet amyloid oligomers strong candidates to be key pathogenic species in the development of disease.

By contrast, oligomeric precursors of amyloid fibrils with parallel \(\beta\)-sheet architecture are likely to accumulate only transiently, as they can elongate rapidly and so generate fibrils. We propose, therefore, that the \(\beta\)-sheet geometry and the rates (and energy barriers) associated with the structural conversions between different \(\beta\)-sheet geometries could play a key role in the process of the misfolding and self-association of amyloidogenic proteins by dictating the kinetic stability and the potential toxicity of the different forms of aggregates (Chen et al., 2015). In addition, our results have revealed an inherent multiplicity in the process of protein misfolding with direct analogies to the multiplicity of parallel pathways observed in the productive folding of a range of proteins (Dinner et al., 2000). The concept that a multiplicity of assembly steps - analogous to those of productive folding - results in an ensemble of oligomers with differing \(\beta\)-sheet arrangements, rates of elongation and inherent toxicities leads to the interesting possibility that protein misfolding and aggregation process in the cell can generate species with different pathological roles depending on the nature of the stochastic processes occurring during the self-assembly process. In addition, the elongation prone, fibril-like oligomers with parallel \(\beta\)-sheet arrangement could act as key

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**Fig. 5. Representation of a putative energy surface for the amyloid aggregation process.** The energy landscape for the formation of amyloid aggregates is likely to be rugged and to be characterized by large numbers of degenerate energy states with significant energy barriers between different regions of conformational space. The simplified free-energy surface shown has been taken from the folding of hen lysozyme (Dinner et al., 2000) to illustrate the analogies between productive folding and misfolding and aggregation. The consequence of such a landscape is the existence of multiple pathways each with distinct oligomeric species. Some pathways will generate oligomeric species that are only transient and that rapidly elongate and convert into fibrils (e.g., the pathway depicted in yellow). But there are other pathways that generate oligomeric species that are trapped in local minima and, therefore, accumulate (e.g., the pathway depicted in red). These oligomers present a structural configuration that is not optimal for elongation and therefore could only transform into fibrils after major structural rearrangements that are likely to involve partial unfolding and also to be slow (dashed red arrows).
pathogenic species for the spreading and transmission of the disease, whereas long-lived and protease-resistant oligomers with an antiparallel β-sheet arrangement could accumulate within cells and act as potent toxins (Chen et al., 2015).

Our studies of αS, and those of Aβ by other groups (Bodani et al., 2015; Liu et al., 2015), suggest, therefore, that amyloid oligomers can form different structures or strains depending on how they acquire structure during the early stages of the self-association process, and that different strains of oligomers can generate differing degrees of toxicity. Indeed, it has recently been found in vivo that two major classes of Aβ oligomers exist: one type appears likely to possess predominant parallel β-sheet structure and remains confined to the vicinity of the plaques and does not impair significantly cognitive abilities. The other type seems likely to possess anti-parallel β-sheet structure, and is formed before the plaques are observed and is location more highly dispersed. In line with the conclusions drawn from our results, this last type of oligomer has been suggested to have greater potential to cause global neuronal dysfunction (Liu et al., 2015).

In addition, recent evidence suggests that different types of fibrillar strains or polymorphs of αS have different seeding capabilities (Guo et al., 2013) and present different abilities to propagate, inducing variable neuronal vulnerability and pathology (Boussert et al., 2013; Heise et al., 2005; Peelaeerts et al., 2015; Prusiner et al., 2015), suggesting a potential link between the structure and pathology of different αS fibrillar strains. Indeed, different fibrillar polymorphs or strains of αS have been related to different αS-related pathologies such as PD, dementia with Lewy bodies and multiple system atrophy (Peelaerts et al., 2015; Prusiner et al., 2015). Interestingly, the variability in amyloid polymorphs of a given polypeptide has also been related to different stages in the progression of a given amyloid disease (Thal et al., 2006).

In summary, recent studies of protein aggregation support the idea that multiple aggregated species can be generated through diverse misfolding pathways during the process of amyloid aggregation of a given polypeptide chain, and also possess different degrees of neuronal toxicity. For example, amyloid fibrils with parallel in-register topologies are particularly efficient recruiting amyloidogenic proteins through a self-templating process as a result of the particular properties of the growing fibril ends. In contrast, we have found that oligomers that do not elongate rapidly into fibrils are intrinsically more highly toxic than their fibrillar counterparts when neuronal cells are exposed to either of these species (Chen et al., 2015). This finding is in line with initial observations in the prion field, in which a clear distinction between the intrinsically toxic species and the species that propagates pathology has been suggested (Sandberg et al., 2011). It, therefore, seems reasonable that different types of aggregates populated during the process of self-assembly of a given protein could be involved in different stages of the development of a particular pathology, some species being directly involved in the induction of neurotoxicity and others in the propagation of pathology (Chen et al., 2015).

The ability to identify the structural features of the specific species involved in the distinct pathological processes would not only provide important insights into the mechanisms of the generation of toxicity but could also be of great value in the design of effective therapeutic and diagnostic approaches that rely on specific protein conformations. For example, we could potentially design antibodies or small molecules that target specific protein species in order to inhibit specific steps that target species of protein aggregation in order to block specific steps in the process of aggregation, to reduce the ease of conversion of the protein into pathological conformations, to reduce the toxicity of the pathogenic protein species or to increase their selective clearance. Two examples of small molecules with potential therapeutic interest have been recently reported following these ideas: one representing an approach in which small molecules can be designed to inhibit specific steps in the aggregation reaction (Habchi et al., 2016a, b), and another representing an approach in which the oligomer toxicity was in addition significantly reduced by binding to a small molecule (Perni et al., 2017).

Importantly, and in the light of the recent findings, combination of methods that target simultaneously or sequentially different protein species might be needed to prevent fully the toxicity associated with the multiple pathogenic processes that occur during the development and progression of the disease.

In general, fundamental biophysical and structural studies of protein aggregation are already contributing to the development of new approaches to identify effective strategies for the treatment of amyloid disease. The combination of therapies targeting different forms of toxic amyloid aggregates of a given polypeptide chain, perhaps alone or in combination, could together target a range of key pathological pathways in these diseases.

To achieve this objective, a detailed understanding of the conformational species that are particularly toxic under different conditions will be a key factor in the effective development of targeted drug therapeutics.

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