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Mini review

Peptide inhibitors of amyloid aggregation

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Summary. Amyloid aggregation is involved in the pathogenesis of various human diseases, primarily neurodegenerative, but also metabolic and other systemic diseases. Current therapies are mostly symptomatic treatments that do not address the underlying cause of the condition. To develop effective therapeutics, it is crucial to understand the mechanism of fibril formation. The amyloid fibrillation cascade is complex and involves three processes: primary nucleation, secondary nucleation, and elongation (with fragmentation). This process is characterized by a repertoire of intermediate states, which are potential targets for therapeutics. Special emphasis is placed on oligomeric and fibrillar forms, as they cause the most harm within the organism due to their toxicity. Studying the mechanism of amyloid formation common to various disease-related intrinsically disordered proteins is thus fundamental for therapeutics development and identifying novel diagnostic markers among these intermediate states. Consequently, biochemical studies of amyloid aggregation, particularly those monitoring kinetics and structure of aggregating species, are extensively conducted. Some of the most promising therapeutics for Alzheimer's disease and similar amyloid-related conditions appear to be peptide-based, either antibodies designed to target specific states in the aggregation process, chaperones with broad substrate specificity and specific thermodynamic properties, or small peptides that facilitate drug delivery. In addition to novel therapeutic strategies, early diagnosis is crucial for addressing amyloid diseases. This paper gives an overview of the mechanism of amyloid formation common for different disease-related intrinsically disordered proteins, and the potential for peptide therapeutic development that arises from a mechanistic approach – monitoring aggregation kinetics.

Keywords: aggregation kinetics, amyloid antibodies, amyloid fibrils, chaperones, peptide inhibitors.

AMYLOID GROWTH AND KINETICS

Assembly of peptides into cross-β-sheet structures characteristic of amyloid fibrils has been reported for dozens of proteins. The most extensively studied peptide assemblies are those involved in amyloid-related diseases, especially neurodegenarative diseases - Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, as well as diabetes type 2 (Aguzzi and O'Connor 2010; Knowles et al. 2015). These proteins, among which intrinsically disordered amyloid-β (Aβ), tau, α-synuclein, huntingtin, and IAPP stand out, all have different sequences but form amyloid aggregates with common characteristics: cross-β-sheet structure and diffraction pattern, defined diameter, fibrillar morphology, binding of ThT/Congo Red, and stability, including resistance to proteolysis (Knowles et al. 2014). The mechanism of amyloid fibrillation and its kinetics, when monitored in vitro, proceed through the same phases for most proteins. Understanding the mechanism of this process is essential for developing potential inhibitors, which is the central goal in the amyloid research field, keeping in mind tens of millions of individuals affected with amyloid-related neurodegenerative diseases

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(Dobson 2017). Still, the numbers are ten times higher when diabetes type 2 patients are also included in the statistics. These figures are more than just numbers; they represent individuals experiencing considerable distress due to the severity of their symptoms, which profoundly affect the quality of life and overall well-being of the afflicted.

Amyloid growth monitored in vitro generally follows sigmoidal kinetics, with few exceptions, e.g. globular proteins with well-defined structures that are forced, by using highly destabilizing conditions, to undergo amyloid fibrillation (Milošević et al. 2020, 2021; Mijin et al. 2023). On the other hand, intrinsically disordered proteins undergoing amyloid fibrillation in physiological conditions progress through the standard three phases of sigmoidal kinetics – lag phase, exponential growth, and stationary phase (Fig. 1). The absolute kinetics may vary from minutes to days depending on the amino acid sequence-related energetic barriers on the way. The slowest step hidden in the lag phase is the dynamic process of primary nucleation, consisting of stochastic events of monomer interactions, association, and dissociation, including conformational changes. Particular conformational changes lead to the formation of a nucleus, i.e., multimeric species with a higher rate of growth than the rate of dissociation (Frieden 2007). The lag phase is not stationary as the shape of the curve may imply, but, on the contrary, a constant formation and dissociation of fibrils goes on until a critical amount of fibrils forms, followed by further exponential growth (Knowles et al. 2009). Nucleation processes are essential for fibril formation since they promote the separation of a new phase in the system. Depending on the environment, nucleation can be homogenous or heterogeneous. In the former, interactions occur solely between protein monomers or with the solvent, without any influence from other factors, such as surfaces, which might trigger the nucleation. However, such an environment is hard to imagine, neither in vitro nor in vivo conditions, as there are always surfaces present, such as cell membranes, other macromolecules, and macromolecular complexes (in vivo), or at least the surface of a container (in vitro). Therefore, all nucleation processes in amyloid fibrillation are indeed heterogeneous. Nuclei are not defined species; they are argued to be oligomers with varying numbers of monomers. Increasing the number of residues in a peptide decreases the number of monomeric subunits forming a nucleus, as was reported for poly-Gln peptides (Wetzel 2012). For monomer assembly to become a fast-growing nucleus requires conformational changes, and whether these occur at the monomeric level or afterward, when oligomers form, is still under scrutiny. Anyhow, nucleation is considered a rate-limiting step in the aggregation process (Dear et al. 2020; Michaels et al. 2020). It seems that

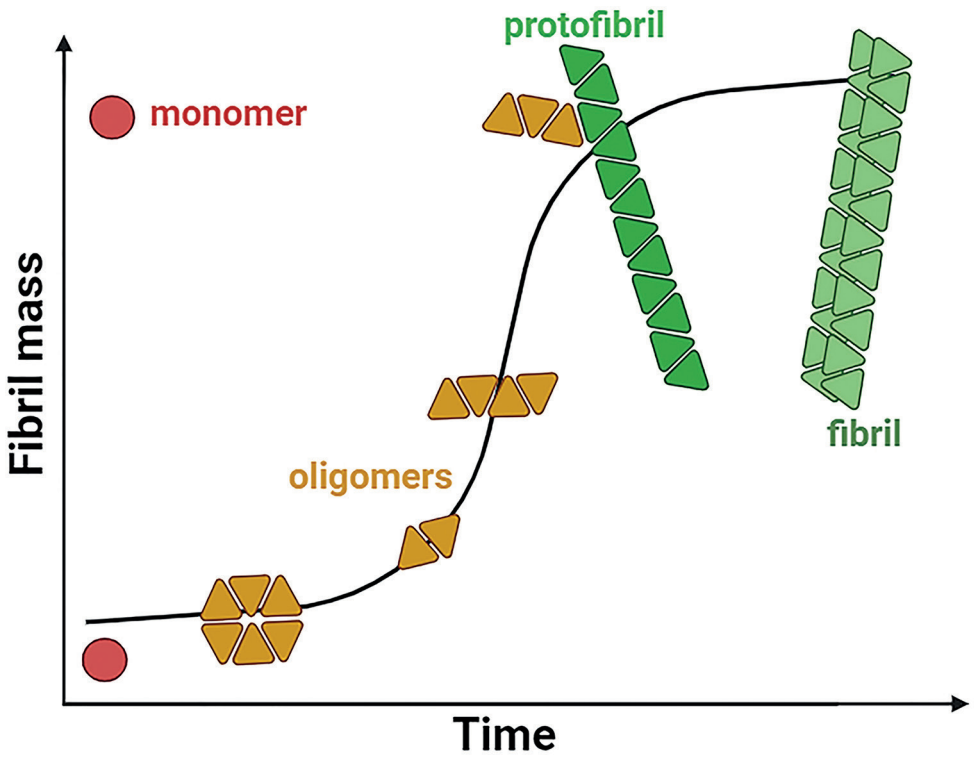

Fig. 1. Kinetics of amyloid formation with a schematic representation of intermediate states. Different species involved in all stages of the aggregation are shown alongside the curve according to the peak of their concentrations in the process.

the higher entropy of a monomeric form supports the thesis that conformational change occurs at this state, but it is challenging to elucidate the fine-tuning of the fibrillation process with the methodology available.

The further extension of the preformed nuclei, i.e., elongation (Fig. 2), also requires conformational changes to fit the monomers into the growing structure. However, the energetic barrier for this transformation appears to be lower, making this step faster (Meisl et al. 2016). Besides the increase in total fibrillar mass due to elongation, there is an increase in the number of fibril molecules, most likely due to fragmentation that occurs above a certain fibril size threshold. Nevertheless, the model with primary nucleation, elongation, and fragmentation has not been the best fit for experimental kinetic data for amyloid-β aggregation. However, introducing secondary nucleation significantly improved the model (Cohen et al. 2013). Although secondary nucleation combines with fibril elongation, the former is predominant owing to its much higher rate (Meisl et al. 2016). During secondary nucleation, which is both fibril- and monomer-dependent, preformed fibrils act as catalytic surfaces that induce conformational changes at specific branching points in fibrils, i.e., where the new monomers attach. This complex process includes both association and conformational changes in an unknown order. Once oligomeric branches detach, they elongate further forming fibrils that serve as new catalysts in the secondary nucleation process. Whether monomers or particular oligomers attach to fibril surface is still unknown. However, there is growing evidence of conformational changes of monomers/oligomers upon the binding which makes the oligomeric branches growing-competent. This process leads to an exponential growth of fibril mass because of the rapid growth of the number of elongating species. The final stationary phase occurs when the system runs out of monomers, or, in the language of thermodynamics, when the chemical potential of all species becomes equal. Experimental evidence of secondary nucleation lies in seeding, a well-known phenomenon in amyloid fibrillation and crystallography (Cohen et al. 2013). Adding a small amount of preformed fibrils as seeds may speed up amyloid fibrillation via concentration-dependent shortening of the lag phase, with the secondary nucleation already driving the process and primary nucleation no longer a rate-limiting step.

As explained, the process of amyloid fibrillation is kinetically complex, with many species in the interplay. The primary species are monomers, oligomers, and fibrillar species, each more likely to be a diverse population rather than a single state (Michaels et al. 2020; Sehlin et al. 2021). Monomers differ only in their conformation, but those differences are crucial for their aggregation into higher-order structures. Some authors recognized the particular conformations of monomers that facilitate their productive packaging into the amyloids and termed them as early amyloid precursors. Literature data supports the role of molten globule as monomeric species undergoing amyloid fibrillation in the case of globular proteins with folded native states (Yamaguchi et al. 2018).

On the other hand, oligomeric states are vaguely defined in the literature; hence, there is a discrepancy in the use of this term. While some authors proclaimed protofibrils to be oligomeric intermediates, others reserved this term for spherical intermediates, which precede fibrillar forms. Varying parameters, including size, the rate of their further growth into fibrils, biological effects and toxicity characterize oligomers (Linse 2017; Dear et al. 2020). The broadest definition of oligomers encompasses all species containing 2 to 30 monomers. The operational definition describes an oligomer as a species having the mass between monomers and fibrils or the species eluting at any volume between the void and monomer-eluting point upon size-exclusion chromatography (Nilsberth et al. 2001; Johansson et al. 2006). Other perspectives differentiate between structurally or functionally distinctive species. Metastable oligomers are considered to be the most toxic species which induce pathological states in misfolding process (Knowles 2014). Even though the cytotoxicity of some oligomers was demonstrated, it does not

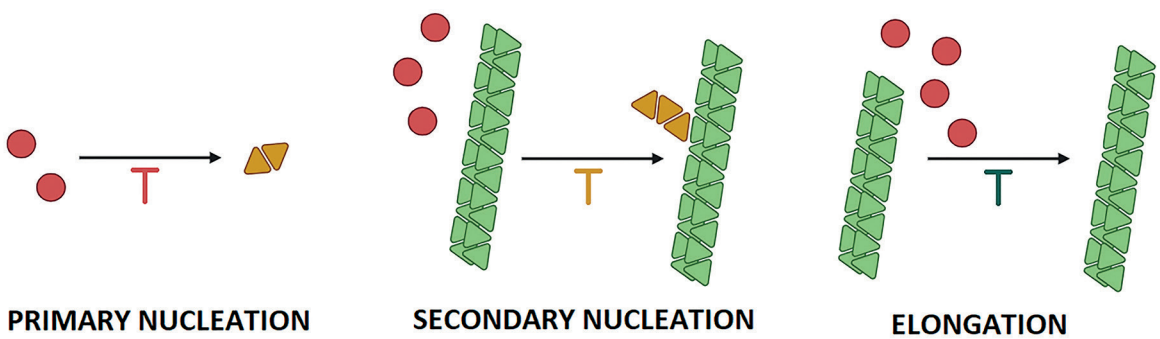

Fig. 2. Phases of amyloid fibrillation and potential steps for inhibition of the process. Red - monomers, yellow - oligomers, green - fibrils.

mean any oligomeric assembly of amyloidogenic proteins will be cytotoxic. A recent theoretical study brought a view of oligomers with a high off-rate undergoing conformational changes into productive, fibrillar, or growing-competent oligomers, which, in turn, quickly transform into a fibrillar state (Dear et al. 2020). Therefore, the oligomeric state is the least understood state of all three, owing to its low stability and fast transition to monomers or fibrils for non-productive or productive oligomers, respectively. Being elusive and hard to work with (experimentally), oligomers remain vaguely defined and these different populations have been only theoretically described. Moreover, whether productive and non-productive oligomers are correlated with their toxicity can be a subject of discussion.

Fibril populations differ from pre-fibrillar species by their elongated morphology and extensive β-sheets perpendicular to the fibril axis. Any particular fibril population is broad, encompassing short filaments, protofibrils, and a mature fibrillar state (Fig. 1). Protofibrils are usually defined as rod-like and worm-like filamentous late-stage intermediates lacking periodicity in their structure (Kodali et al. 2007; Buell et al. 2014), although with structural resemblance to fibrils (Walsh et al. 1997). Protofibrils assemble into protofilaments, which represent single-stranded fibrils helically twisting into mature fibrils. Mature fibrils vary in size, and while shorter forms can be less than 100 nm long, longer ones exceed 1 μm (Gade Malmos et al. 2017). Common characteristics that classify these structures as amyloid fibrils include a diameter of about 10 nm, a high level of β-sheet structure, a cross-β diffraction pattern, and a core resistant to hydrogen exchange. Nevertheless, mature fibrils made from the same peptide may acquire various structures depending on aggregation conditions (Pálmadóttir et al. 2023).

DIFFERENT PATHWAYS OF INHIBITION

Owing to the complexity of the aggregation described, which involves numerous distinctive processes, the number of inhibition targets is great. Primary nucleation, elongation, secondary nucleation, or fragmentation are all possible targets for intervention (Fig. 2). The shape of inhibition curves obtained by monitoring the aggregation kinetics in response to the rising inhibitor concentrations and fitted using the Amylofit platform (http://www.amylofit.ch.cam.ac.uk), enable deciphering the inhibited process. Inhibition of primary nucleation is characterized by the extension of the lag phase (Fig. 3). The inhibition of secondary nucleation or elongation both affect the slopes of inhibition curves, as these processes dominate in the exponential phase (Fig. 3).

Particular processes in the aggregation have different reaction orders meaning that concentration of one or multiple species can affect the reaction rate. Those species are potential targets for inhibition of a particular step. Since the primary nucleation is monomer-dependent, the inhibitors of this step should bind to monomers or early oligomer states. Potential inhibitors should be designed to sequester the entire population of monomers to prevent their aggregation (Markovic et al. 2024). Alternatively, binding to early oligomers could be more efficient, since their concentration makes up less than 1% of the entire protein content, as shown for Aβ (Michaels et al. 2020). Considering elongation inhibition, the amount of inhibitor required is significantly lower, as this process occurs only on fibril ends. Blocking fibril ends when fibril concentration is low, i.e., in cases of early administration of inhibitors, would require much fewer inhibitor concentrations compared to primary nucleation inhibition. Secondary nucleation is dependent on both fibrils and monomers and involves the formation and detachment of branches in the form of oligomers, hence, either of these species could be targeted to prevent further aggregation. One approach is to find binders specific for fibrils, while another is to target oligomers, which might be more effective as already mentioned. Off note, the concentration of oligomers further decreases after the midpoint of the aggregation (Michaels et al. 2020). As the dominant process in the exponential phase of aggregation, many authors think inhibiting secondary nucleation is the best approach for treating amyloid-related diseases (Michaels et al. 2020; Chia et al. 2023). Interestingly, it was shown that antibodies designed specifically against monomers would have the smallest potential to inhibit aggregation, and, vice versa, potential antibodies that could recognize and bind all other species in the system (oligomers, protofibrils, and fibrils) would have higher inhibitory potential; this was the result of quantitative mathematical modeling of Alzheimer's disease progression, assuming it is a non-linear process (Markovic et al. 2023). Therefore, the quest for monomer binders to inhibit amyloid aggregation is misleading. In contrast to fibrils, soluble oligomers and protofibrils are cytotoxic, and they can affect synapses, inducing memory loss, as demonstrated in many studies (Nilsberth et al. 2001; O'Nuallain et al. 2010; Goure et al. 2014). Protofibrils, for example, are found to enter microglia to a higher extent than monomers (Gouwens et al. 2016). This perspective further supports the importance of inhibiting the secondary nucleation as this process is the source of new oligomer intermediates.

Some authors believe that mature fibrils are completely inert, so that expediting transition from oligomeric soluble (and thus diffusible) species into fibrillar end-state species would decrease the cytotoxic effect. Not only does this hypothesis lack evidence, but fibrils and plaques were also found to be in correlation with the synaptic dysfunction in AD (Le et al. 2001; Meyer-Luehmann et al. 2008; Spires-Jones

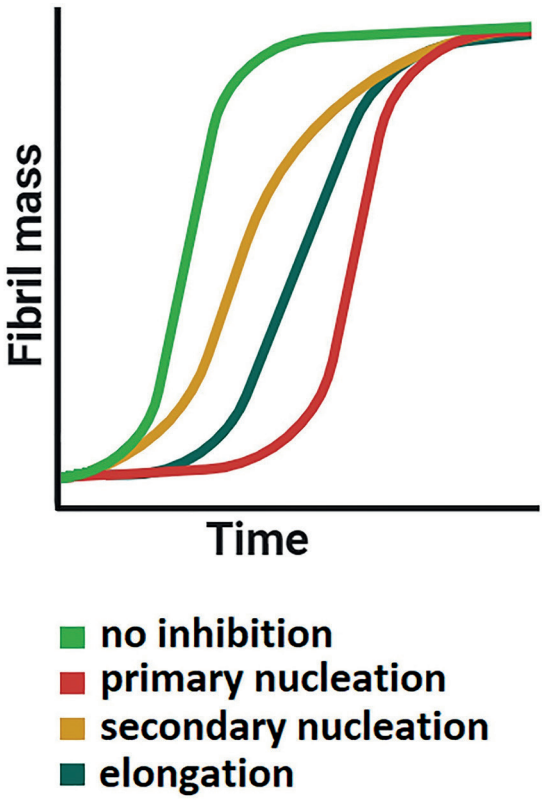

Fig. 3. Amyloid aggregation kinetics in the presence of inhibitors of primary nucleation, secondary nucleation and elongation.

and Hyman 2014). Apart from the finding mentioned above, the inertness of fibrils is not in accordance with their formation mechanism. Given that secondary nucleation is the dominant step in fibril formation, not only do they represent the end state, but they also promote fibril formation in a sort of positive-feedback loop. In that sense, the accumulation of fibrils further speeds up the aggregation process and promoting fibril formation is not a reasonable approach to the treatment of amyloid-related diseases.

PROTEIN AND PEPTIDE INHIBITORS

During the last few years, the efficacy of protein-based therapeutics in treating various diseases has been proven, increasing the percentage of their approval to over 20% annually (Al Shaer et al. 2022). Treatment of amyloid-related diseases is not an exception, so most drugs in clinical trials are peptide-based, primarily antibodies and their fragments. For example, three antibody-based drugs were approved by the US Food and Drug Administration in the last four years for AD treatment following decades without a single AD drug approval. Table 1 displays the inhibitory mechanism of these drugs and their specific targets. Monoclonal antibody Solanezumab, that has been investigated as a neuroprotector for patients with AD, for example, binds monomers only. However, the clinical trials showed that this antibody was not efficient in slowing down AD progression (Doody et al. 2014b; Honig et al. 2018; Salloway et al. 2021). In contrast, higher curative potential of antibodies targeting oligomers, protofibrils, or fibrils was demonstrated by both clinical trials and mathematical modeling. So far, Lecanemab, monoclonal antibody raised against protofibrils (Johannesson et al. 2024), has been one of the most promising AD therapeutics.

The catalytic nature of fibrils facilitates their formation; hence, successful therapy should enable their removal. Mathematical modeling indicated that antibodies with effector function would be the most efficient in decelerating AD progression. Two monoclonal antibodies designed to treat AD, Aducanumab and Bapineuzumab, possess fragment crystallizable regions (Fc) that interact with Fc receptors, thus triggering a microglial response (Hladky and Barrand 2014). Unfortunately, this approach is associated with severe side effects (Withington and Scott Turner 2022). Donanemab, which binds to plaques, has gotten an approval for AD treatment in 2024 due to its premium efficacy over other antibody-based therapeutics. It is an IgG1 monoclonal antibody that recognizes the modified Aβ version found in plaques (Rashad et al. 2023). To sum up, the future of passive immunotherapies lies in optimizing the specificity of antibodies for oligomers, protofibrils, and fibrils, and also in optimizing the effector functions of antibodies to accelerate the clearance of toxic soluble species and fibrils.

Whereas antibodies are specific for a certain amyloid protein, chaperones are natural peptides able to inhibit formation of amyloids from different precursor proteins. So far, the pharmacological potential of chaperones has been extensively investigated in preclinical studies and shows promising inhibitory activity. Brichos domain is an efficient inhibitor (Cohen et al. 2015) of secondary nucleation of α-synuclein and amyloid-β, with specificity toward fibrils. DNAJ proteins (DNAJb6 and DNAJb8) also prevent aggregation of different proteins into amyloid fibrils, the first one studied being polyglutamate (Gillis et al. 2013), but also proven for amyloid-β (Månson et al. 2014) and α-synuclein (Aprile et al. 2017).

Current drug discovery tendencies favor using small peptides instead of entire proteins. This approach has many advantages including more efficient delivery due to better tissue penetration, higher drug stability, and fewer off-target interactions. A phage-display approach has been recently employed in screening peptide inhibitors of Aβ amyloid aggregation with calbindin as an inert scaffold. The most potent oligopeptide-based binders were selected, synthesized, and

Table 1. Approved antibody therapeutics for Alzheimer's disease (AD).

Therapeutic	Process inhibited	Target	
Aducanumab	Secondary nucleation	oligomers/fibrils/plaques	Linse et al. 2020
Lecanemab	Unknown	protofibrils	Johannesson et al. 2024
Donanemab	Unknown	plaque	Rashad et al. 2022

tested using Surface plasmon resonance. The study selected a peptide with sequence YILTRIM that binds specifically to oligomers, thus preventing secondary nucleation (Linse et al. 2022). Further studies in that direction have been conducted worldwide with great expectations for their efficiency.

CONCLUSION

Searching for drugs that efficiently halt amyloid fibrillation of various disease-related proteins is a long-lasting race, which is, fortunately, speeding up with the development of high throughput screening methods. Besides targeting the suitable species and expediting the effector functions of therapeutical antibodies, which were discussed in our review, another challenge lies in developing an efficient drug delivery system. Despite all the hurdles in the process of peptide-based drug development, they have been extensively investigated because of their great curative potential. However, this is only one direction of research on amyloid diseases. A lot of effort is invested in developing early diagnostics, which is essential for amyloid-related disease prevention, especially knowing that the timescale of aggregation *in vivo* significantly surpasses that in vitro. The aggregation kinetics in vivo are intrinsically difficult to decipher, although we know that the onset of clinical symptoms in AD appears decades after the first pathophysiological aggregation processes.

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