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REVIEW

Thermodynamic and kinetic approaches for drug discovery to target protein misfolding and aggregation

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ABSTRACT

Introduction: Protein misfolding diseases, including Alzheimer's and Parkinson's diseases, are characterized by the aberrant aggregation of proteins. These conditions are still largely untreatable, despite having a major impact on our healthcare systems and societies.

Areas covered: We describe drug discovery strategies to target protein misfolding and aggregation. We compare thermodynamic approaches, which are based on the stabilization of the native states of proteins, with kinetic approaches, which are based on the slowing down of the aggregation process. This comparison is carried out in terms of the current knowledge of the process of protein misfolding and aggregation, the mechanisms of disease and the therapeutic targets.

Expert opinion: There is an unmet need for disease-modifying treatments that target protein misfolding and aggregation for the over 50 human disorders known to be associated with this phenomenon. With the approval of the first drugs that can prevent misfolding or inhibit aggregation, future efforts will be focused on the discovery of effective compounds with these mechanisms of action for a wide range of conditions.

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Protein misfolding; protein aggregation; protein phase separation; amyloid aggregates

1. Introduction

The phenomenon of protein misfolding and aggregation has been associated with over 50 degenerative diseases, collectively known as amyloidoses [1–3]. These conditions include neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, systemic disorders such as light chain (AL) amyloidosis and hemodialysis-related amyloidosis, and localized disorders such as type II diabetes [1–3]. The proteins that misfold and aggregate in these diseases are unrelated in sequence, structure and function [1,4]. Some proteins, including amyloid β (Aβ) [5,6] and tau [7,8] in Alzheimer's disease, α-synuclein in Parkinson's disease [9,10], and IAPP in type II diabetes [11,12], are natively disordered. Other proteins, including antibody light chains in amyloid light-chain amyloidosis [2,13], β_2-microglobulin in hemodialysis-related amyloidosis [14] and transthyretin in familial amyloid polyneuropathy [15,16] are folded in their native states.

Although the clinical manifestations of these diseases cover a vast range of symptoms, they share a common molecular signature, as they are associated with the presence of amyloid aggregates in the affected tissues and organs [1–3,17]. The amyloid state is characterized by the formation of long fibrillar assemblies with a core structure composed of β-sheets whose strands run perpendicular to the fibril axis [1,17], and stabilized by a network of backbone hydrogen bonds [18]. The advent of cryo-electron microscopy has recently enabled the determination of patient-derived high-resolution amyloid structures associated with many different protein misfolding diseases, revealing the existence of different disease-specific amyloid folds, often referred to as fibril polymorphs [19–22].

In order to develop effective treatments based on amyloid aggregation as a target, one should understand the molecular origins of this process, as well as characterize and quantify the most cytotoxic aggregated species produced during the formation of the amyloid state [1–3,6,23,24]. It has been proposed that supersaturation is the common driving force for the aggregation of these proteins [25,26]. In this view, many proteins are more stable in their amyloid states than in their native states, at least at the concentrations at which they are expressed under normal cellular conditions [25,26] (Figure 1). Under these circumstances, aggregation can be delayed but cannot be completely avoided. Proteins can perform their functions because their native states are kinetically more accessible than the amyloid states, and high free energy barriers slow down the aggregation process [1,27,39]. Furthermore, when they form, protein aggregates are removed by degradation pathways, including the ubiquitin-proteasome and the autophagy-lysosomal systems [40]. More generally, the protein homeostasis system regulates the behavior of proteins in terms of their conformations, interactions, concentrations and localization [41,42], and contributes to the maintenance of the functional state of proteins in the presence of a thermodynamic drive toward aggregation [40]. Upon aging, however, the progressive impairment of the protein homeostasis system may lead to the gradual accumulation of protein deposits, whose presence in turn initiates a cascade of pathological processes [43–49].

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Article highlights

- Protein misfolding and aggregation into the amyloid state is a widespread phenomenon, which, when dysregulated, results in a variety of different diseases.
- At physiological protein concentrations, the amyloid state can be thermodynamically more stable than the native state. Under these conditions, aggregation is inevitable, although proteins should cross high-free energy barriers to aggregate.
- Thermodynamic approaches for drug discovery for protein misfolding diseases are based on the stabilization of the native state with respect to the misfolded and amyloid states.
- Kinetic approaches for drug discovery to target protein aggregation are based on reducing the rate of conversion between the native and amyloid states.
- Thermodynamic and kinetic approaches could be extended to amyloid aggregation within liquid-like condensates generated by protein-phase separation.

Therapeutic interventions could thus be aimed at supplementing the protein homeostasis system as it is progressively impaired upon aging. In broad terms, one could think of two complementary strategies to reduce the driving force toward aggregation, the first based on the thermodynamics and the second on the kinetics of the process (Figure 1). In a thermodynamic strategy, since supersaturation depends on the free energy difference between the native and amyloid states, the aim is to develop compounds that stabilize native states [16,28–32]. In a kinetic strategy, the goal is to increase the height of the free energy barrier between the native and amyloid states. In this second approach, the thermodynamic driving force remains unchanged, but the process is slowed down, so that even a protein homeostasis system of reduced functionality could effectively remove the aggregates [33–38]. We should point out that the optimization of the kinetics of binding of small molecules to their targets, in terms of the balance between on rates and off rates, has a long history in drug discovery [50–52]. However, the type of kinetic strategy that we are concerned with here is specifically aimed at increasing the rate of conversion of native states into aggregates.

In this review, we discuss recent results obtained using the thermodynamic and kinetic strategies against protein aggregation.

2. Thermodynamic approaches

A drug discovery strategy to target protein aggregation based on the stabilization of the native state was first demonstrated

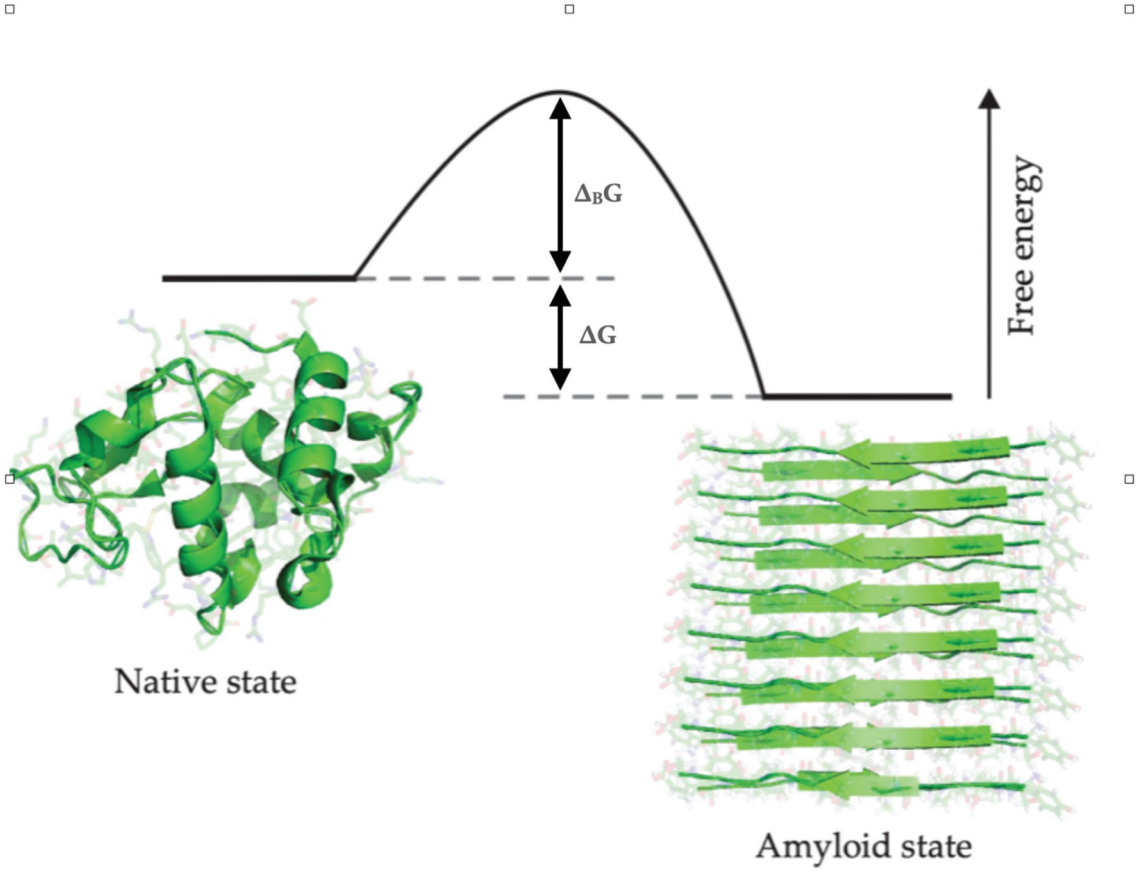

Figure 1. Schematic free energy landscape of protein aggregation. at the concentrations at which proteins are expressed, the amyloid state of proteins can be thermodynamically more stable than the native state [26,27]. Thermodynamic approaches to drug discovery are aimed at stabilizing the native state by reversing the sing of the free energy difference (ΔG) between the native and amyloid states [16,28–32]. Kinetic approaches have instead the target of increasing the free energy barrier for the conversion between the two states ($\Delta_B G$), thus prolonging the lifetime of the native state [33–38]. Thermodynamic and kinetic approaches can be complementary to each other. The former may be more effective at preventing aggregation at low protein concentrations, while the latter may be preferable at higher concentrations where the aggregation process is more rapid. Ultimately, the choice of approach depends on the specific circumstances of the protein aggregation problem at hand. Reproduced from [1] with permission of Springer Nature.

over a decade ago in the case of transthyretin, initially for familial amyloid polyneuropathy, and subsequently for both familial and sporadic amyloid cardiomyopathy [16,28,53,54]. Starting from the observation that the first step in the aggregation pathway of transthyretin is the dissociation of the native homotetramer, the small-molecule tafamidis was identified to stabilize the native complex [28,53].

Based on the example of tafamidis, other compounds with a similar mechanism of action, known as pharmacological chaperones [16,55], have been developed. Six drugs for three other amyloidoses have been recently approved by the FDA: (i) migalastat, which targets destabilized mutant α-galactosidase A in Fabry disease [56], (ii) voxelotor, which targets a mutant form of hemoglobin in sickle cell disease by stabilizing the tetrameric oxygenated form of the protein [57], and (iii) ivacaftor (also known as VX-770) [58], elexacaftor (VX-445) [59] and tezacaftor (VX-661) [60], which act synergistically to target mutant forms of the anion channel cystic fibrosis transmembrane conductance regulator (CFTR) in cystic fibrosis by promoting channel gating (ivacaftor) and by stabilizing the native state (elexacaftor and tezacaftor) [16,59]. Other compounds with a similar mechanism of action are under development. A small molecule in the sterol class has been shown to act as pharmacological chaperone by inhibiting amyloid fibril formation of mutant α-crystallins, and to reverse cataract in mouse models of these mutants [61,62]. Another sterol (lanosterol) was shown to ameliorate cataract in dogs [63], and is currently commercialized as a veterinary drug (Lanomax, https://www.lanomax.com/). Pharmacological chaperones are also being investigated with great promise to target oncogenic mutants of the tumor suppressor protein p53 [64].

By building on these developments, it has been proposed that approaches based on the stabilization of the native states of proteins may be extended to disordered proteins, which until recently have been considered undruggable [29,31,65–69]. Finding effective drug discovery strategies for this class of proteins would have a major impact since thousands of potential targets are disordered proteins, which are involved in essentially all major human diseases [29,67].

The major obstacle to achieve this goal is that disordered proteins do not readily exhibit binding pockets for forming stable interactions with small molecules. Nevertheless, small molecules have been identified to bind disordered proteins using a range of methods, including nuclear magnetic resonance (NMR) spectroscopy, surface plasmon resonance (SPR), biolayer interferometry (BLI), and fluorescence techniques [66,70–72]. It has been suggested that small molecules may induce the folding of a disordered protein, perhaps just to the extent in which well-formed binding pockets are formed [66,73,74]. It is not yet clear, however, whether drug-like affinity and specificity can be achieved through this type of binding mechanism. To obtain higher affinity and specificity, one possibility is to use larger molecules, such as macrocycles, peptides, or antibodies [75–77]. However, for brain diseases, or diseases in which protein aggregation is intracellular, small molecules would remain preferable, as they would more readily cross the blood-brain barrier and the cell membrane.

Another possibility, beyond the strategies based on the existence of binding pockets mentioned above, is to exploit a different binding mechanism, one in which the complex between the protein and the small molecules remains disordered. This mechanism of binding has been observed for protein–protein interactions and protein-nucleic acid interactions [78–80], and it is likely to be responsible for the phenomenon of protein phase separation (PPS), which has been associated with the formation of membraneless organelles [81–85].

The possibility of a disordered binding mechanism for small molecules has been illustrated recently in the case of Aβ, where the small-molecule 10074-G5 was shown to bind and stabilize the disordered native state of this peptide, thus preventing its aggregation [29,31,32] (Figure 2). The nature of the binding was analyzed in terms of lifetimes of the transient contacts between 10074-G5 and Aβ, finding that the interactions were typically shorter than 10 ns, which is a timescale compatible with a diffusion of the small molecule on the fluctuating surface of the disordered peptide. It was also observed that the conformational entropy of Aβ increased upon interacting with 10074-G5, in a mechanism known as entropic expansion [29], suggesting that exploiting this phenomenon may be a potential therapeutic strategy for disordered proteins. We should note, however, that 10074-G5 is not a specific inhibitor of Aβ aggregation, since this compound was originally identified using a yeast two-hybrid assay to target the heterodimerisation of c-Myc, a transcription factor involved in cell cycle progression, cellular growth and metabolism, differentiation, and apoptosis, with its partner Max [86], and shown to act by binding and stabilizing the disordered c-Myc monomer [66].

By using NMR spectroscopy, another small molecule, fasudil, has been reported to bind the disordered native state of α-synuclein and inhibit its aggregation [87]. The disordered nature of the complex between fasudil and α-synuclein has been described using molecular dynamics simulations, which revealed that fasudil shuttles between transient networks of side-chains, thus forming short-lived contacts with the protein [65]. More recently, an analysis of these simulations indicated that the binding increases the entropy of α-synuclein [88], consistently with the entropic expansion mechanism of disordered binding [29]. We note that the binding of a small molecule to a disordered protein shifts the populations of its substates [71]. This population shift always underlies an entropic expansion, but it could also leave the entropy unchanged, or reduce it [30].

The disordered N-terminal transactivation domain (NTD) of the androgen receptor (AR), a nuclear receptor involved in most prostate cancers [89], was recently targeted with a series of bisphenol A derivatives, one of which, known as EPI-7386, is currently in phase I clinical trials [90]. The mechanism of binding of two of these derivatives, EPI-002 and EPI-7120, was studied using NMR spectroscopy and molecular dynamics simulations. The two compounds were observed to promote the formation of transient local α-helical structures. Although the bound state remains highly heterogeneous, the modulation of its conformational behavior upon binding is sufficient to prevent its interaction with the transcriptional machinery required for elevated AR transactivation in prostate cancer patients [70].

Taken together, these results indicate that small molecules could be found against the thermodynamic driving force

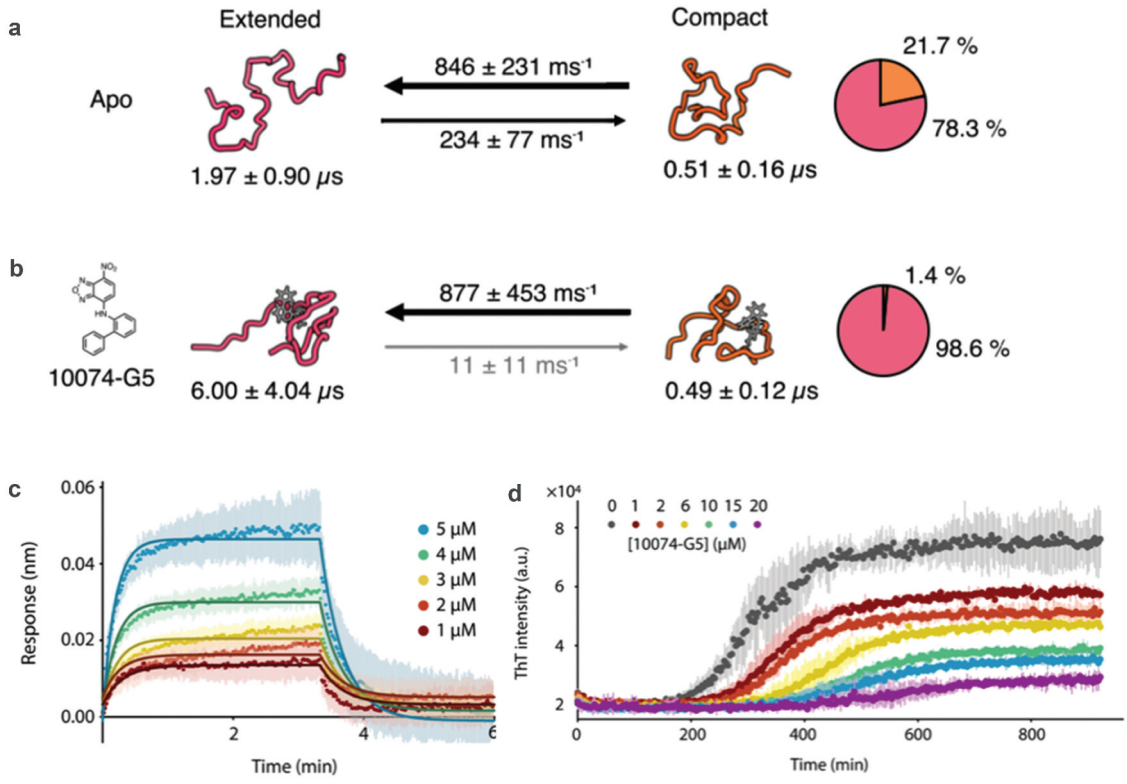

Figure 2. Illustration of a thermodynamic approach. (A) the small molecule 10074-G4 binds Aβ in its monomeric disordered state [31]. (B) The binding increases the population of this state with respect to more compact, partially structured states on pathway to aggregation. Complex formation takes place through a 'disordered binding mechanism,' since both the peptide and the small molecule remain highly disordered in the bound state [31,32]. Panels a and B are adapted from [31] with permission of American Chemical Society. (C) The dissociation constant can be estimated by surface plasmon resonance (SPR) to be around 6 μM. (D) When present in an in vitro aggregation experiment, 10074-G5 increases in a concentration-dependent manner the amount of monomeric Aβ at the end of the aggregation reaction. Panels C and D are adapted from [32] with permission of the American Association for the Advancement of Science Publishing Group.

toward protein aggregation. These small molecules are rather effective in solubilizing the native states of proteins, thus reducing their aggregation. At present, however, in the case of disordered proteins, it remains to be established whether it will be possible to obtain in this way small molecules with sufficient affinity and specificity for their targets to be taken forward in drug discovery programs.

3. Kinetic approaches

Kinetic strategies, which are based on the reduction of the rate of aggregation of proteins, require compounds that increase the height of the free energy barrier between the native and amyloid states. A major challenge of this approach is that the one-dimensional free energy barrier shown in Figure 1 is a simplification. More accurately, the macroscopic rate of aggregation is the result of the combination of the rate constants of a complex network of different microscopic processes [33,91,92] (Figure 3A). These microscopic processes include: (i) primary nucleation, which generates disordered oligomeric intermediates from monomeric reagents, (ii) oligomer conversion, which produces ordered oligomers with the characteristic cross-β structure of amyloid fibrils, (iii) elongation, in which amyloid fibrils grow by monomer addition, and (iv) secondary nucleation, where the surfaces of existing amyloid fibrils catalyze the formation of new oligomers [59,95] (Figure 3A).

In this kinetic network, the aggregating proteins are simultaneously the reactants (the monomers), the products (the amyloid fibrils), the intermediates (the oligomers) and the catalysts (the amyloid fibril surfaces). It is thus important to identify the specific microscopic steps that contribute most to the generation of cytotoxic species [96,97]. In some cases, for example, in amyloid light chain amyloidosis, the cytotoxic species are the amyloid fibrils [2,13], while in other cases, for example, for Aβ in Alzheimer's disease, the most dangerous species are the oligomeric intermediates [1–3,6,23,24]. The non-linear and interconnected nature of the kinetic network underlying Aβ aggregation makes it difficult to predict the effects of compounds that change the rate constants of specific microscopic steps on the production of oligomers [97] (Figure 3B, C). For example, reducing specifically primary nucleation delays the production of oligomers, but it may not change the total amount of oligomers produced at the end of the reaction. Drugs that inhibit primary nucleation would thus be preventative [34].

It is a major challenge to translate macroscopic measurements on the amounts of amyloid aggregates into estimates of the number of oligomers produced, and hence of whether a drug candidate could be expected to reduce the oligomer-induced toxicity. This is illustrated by the case of compounds that reduce the elongation rate. These compounds delay the formation of amyloid fibrils, but increase the amount of

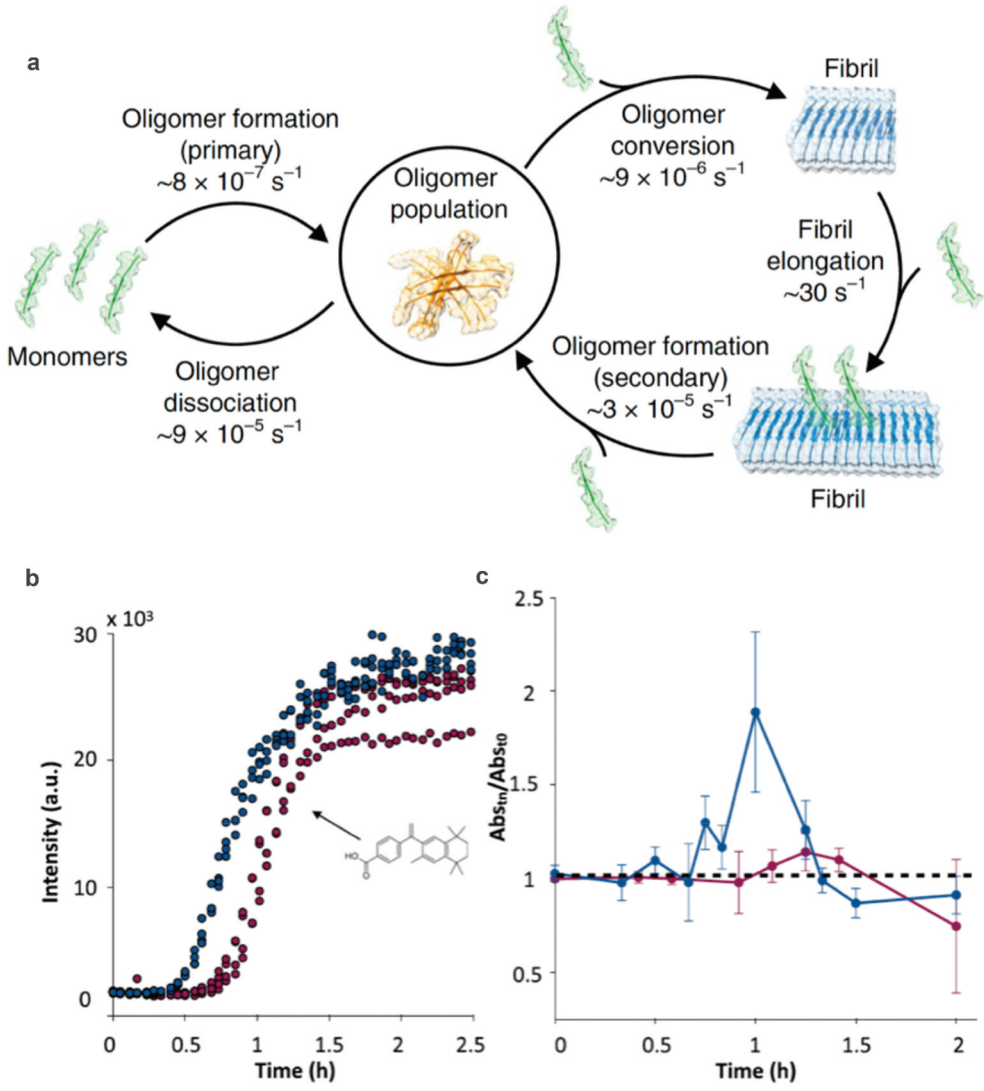

Figure 3. Illustration of a kinetic approach. (A) The free energy barrier in Figure 1 is the result of a complex network of microscopic processes, including primary nucleation, oligomer conversion and dissolution, elongation and secondary nucleation. Adapted from [93] with permission of Springer Nature. (B,C) Small molecules that act on these microscopic processes can delay the formation of amyloid fibrils (B), and reduce the number of oligomers produced during the aggregation reaction (C). Panels B and C are adapted from [94] with permission of the National Academy of Sciences.

oligomers produced during the reaction [97]. This is because the amyloid fibrils act as a sink for monomers. Preventing monomers from binding the fibrils redirects them toward the formation of oligomers. The recent failure in the clinical trials of gantenerumab, an antibody developed to target Aβ aggregation [98], may at least in part be rationalized in this way [96].

Since in the case of Aβ and α-synuclein it has been shown that secondary nucleation is the microscopic process that is most effective in producing oligomers [93,99,100], effective compounds could reduce the rate of secondary nucleation [35,36,101]. One way to do this is to use compounds that bind the catalytic sites on the surface of amyloid fibrils. The molecular chaperone Brichos has been shown to act specifically in this way [102]. The antibody aducanumab, the first disease-modifying drug approved by the FDA for Alzheimer's disease [103,104], in addition to its normal effector function, has been shown to have this mechanism of action [96].

Strategies that exploit structure-based drug discovery methods can be applied to the identification and optimization of compounds that bind structured pockets on the surface of amyloid fibrils. If these binding pockets are the catalytic sites for secondary nucleation, the compounds can be effective inhibitors of both amyloid aggregation and oligomer production, as shown recently in the case of α-synuclein aggregation [38]. Machine learning methods are emerging as a promising route for the discovery of potent compounds with this mechanism of action [105,106].

4. Protein aggregation within protein condensates

In the last decade or so, evidence has been accumulating that in addition to the native and amyloid states, proteins can populate a dense, liquid-like state, known as the droplet state, through protein phase separation [81–85,107,108]. The presence of the droplet state opens the possibility of an

alternative pathway to amyloid aggregation, which goes through liquid-like condensates [109–115] (Figure 4A). This process is known as the condensation pathway, while the direct formation of amyloid aggregates from the native state is known as the deposition pathway [116]. The formation of Lewy bodies in Parkinson's disease may take place through either pathway [116]. In ALS, the progressive maturation of liquid-like condensates of FUS and TDP-43 into gels and amyloid aggregates may generate pathological processes either directly through cytotoxic effects of the aggregates or indirectly by preventing the dissolution of the condensates after they have completed their physiological functions [111–115,118].

While many therapeutic opportunities to target the protein phase separation process may be pursued [85,119,120], here we comment on those that concern the inhibition of the amyloid aggregation process within liquid-like condensates. Initial evidence indicates that the type of kinetic models developed for the deposition pathway may be applicable also to the condensation pathway [117] (Figure 4B). Therefore, the kinetic approach for drug discovery, which was originally developed for the deposition pathway, may conceivably be extended also to the condensation pathway. The thermodynamic approach should also be applicable, since the stabilization of the native state through the binding of small molecules to the native state should reduce the populations of both the condensed and the amyloid states.

5. Conclusions

Although the process of protein misfolding and aggregation remains a challenging target for drug discovery, major advances have been made in recent years, in particular through the validation of the amyloid hypothesis [5,6,104,121,122]. With the first disease-modifying drugs

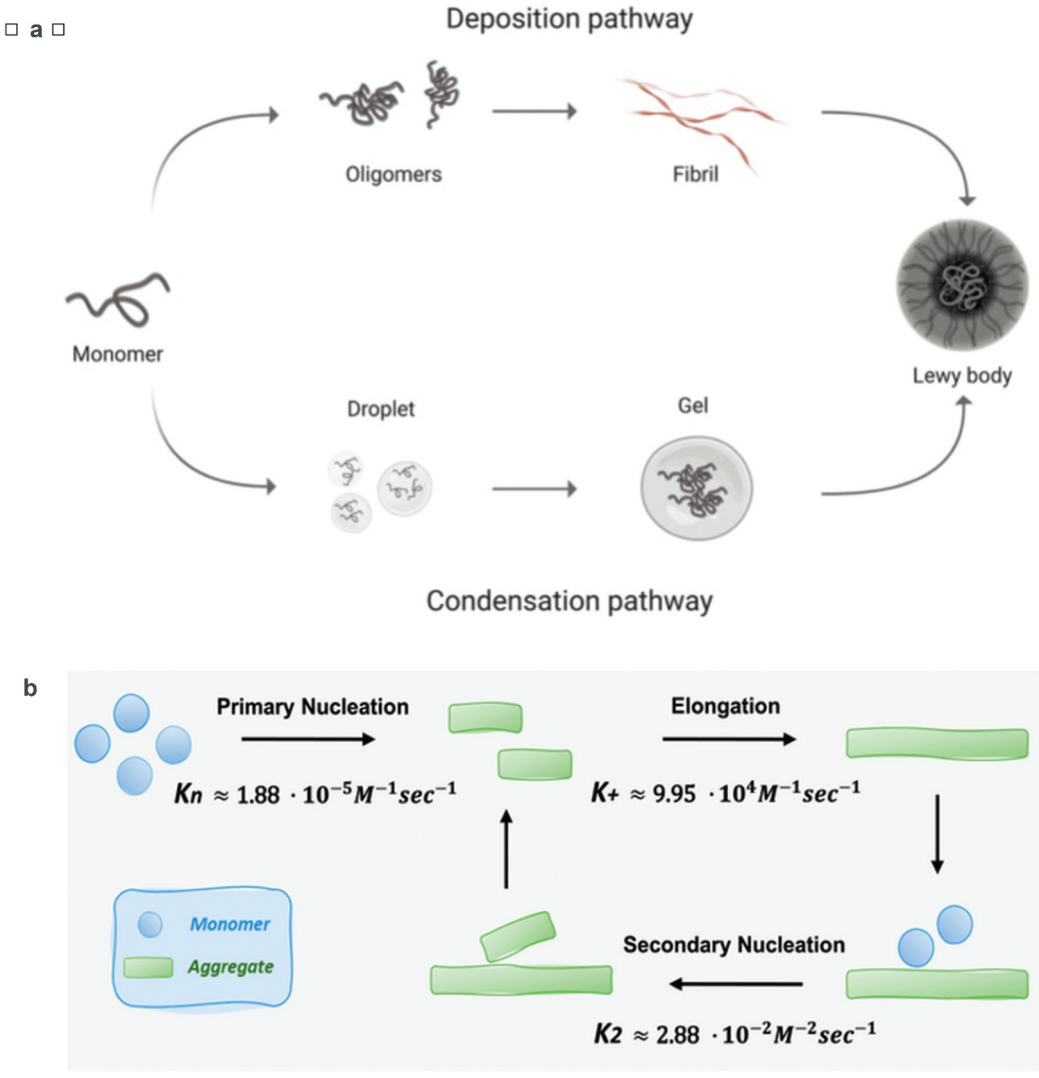

Figure 4. Deposition and condensation pathways in α-synuclein aggregation leading to Lewy body formation. (A) The formation of the amyloid state directly from the native state is known as the deposition pathway, while when it happens through the droplet state is referred to as the condensation pathway. Adapted from [116] with permission of Oxford Academic. (B) In the case of α-synuclein, the kinetic network shown in Figure 3A for the deposition pathway appears to model also the condensation pathway. However, because of the much higher concentration of α-synuclein within condensates, both primary and secondary nucleation are much faster and can be observed in the absence of lipid membranes and at physiological pH along the condensation pathway. Adapted from [117] with permission of the National Academy of Sciences.

having now entered clinical use, the development of more effective compounds with similar targets and mechanisms of action is under way. Both thermodynamic approaches, based on the stabilization of native states [28,53–60], and kinetic approaches, based on the reduction of the rate of aggregation [36,96], are offering powerful routes for establishing successful therapeutic interventions.

6. Expert opinion

There is an urgent need for effective drugs for protein misfolding diseases, which are still largely incurable despite being among the primary causes of disability and death worldwide [123–126]. Drug discovery for these conditions has been extraordinarily challenging, with very few disease-modifying drugs approved to date, and extremely high attrition rates [103,122,127,128]. This situation has been caused, at least in part, by an incomplete understanding of the mechanisms of toxicity in these diseases, and by a lack of effective tools for developing compounds capable of preventing, delaying, or reversing the aggregation process.

On the bright side, the first drugs for protein misfolding diseases are already in clinical use. To date, there are six approved compounds with a mechanism of action based on thermodynamics (Figures 1 and 2) (tafamidis [28,53], migalastat [56], voxelotor [57], ivacaftor [58], elexacaftor [59] and tezacaftor [60]). Two antibodies, aducanumab [103,104] and lecanemab [122], have been approved by the FDA for Alzheimer's disease, which, in addition to their effector functions, are also likely to act through a kinetic mechanism of inhibition of protein aggregation (Figures 1 and 3) [96,121]. These antibodies provide an example of compounds that reduce the proliferation of Aβ oligomers by inhibiting secondary nucleation [96], and slow down the rate of cognitive decline [122]. The approval of these two antibodies offers support for the amyloid hypothesis [5,6], thus prompting renewed efforts in drug discovery programs based on this concept. The development of small molecules with a similar mechanism of action of aducanumab and lecanemab may result in fewer adverse reactions, and, in the case of intracellular aggregates, such as tau tangles and α-synuclein Lewy bodies, in better target engagement.

To extend these advances to other protein misfolding diseases, it will be important to develop more accurate methods for the identification and the quantitative measurements of the most toxic species produced when proteins misfold and aggregate. This is a challenging task, since the aggregation process involves many interconverting species, which are difficult to separate and characterize individually within a complex kinetic network [93,95,96,129]. This task is intertwined with the development of pre-clinical toxicity assays that recapitulate the pathological mechanisms relevant in a disease. Neuronal cell models derived from induced pluripotent stem cells hold great promise in this direction [130–132], as also indicated by the recent revision of the FDA guidelines concerning the use of animal models.

Diagnostic methods, in particular, based on biofluids should be developed in parallel to drug discovery [133]. These methods are essential to assess the clinical efficacy of treatments, particularly those aimed at reducing the amounts of oligomers produced during an aggregation reaction, which are highly elusive species [129]. Accurate oligomer detection and quantification methods are also going to be necessary in pre-clinical target engagement studies [94,96,99,129]. Such methods are also required for the systematic optimization of the potency of compounds during the early stages of drug discovery, where structure–activity relationships should be developed [36,93].

Moving forward, the introduction of machine learning methods is going to facilitate the drug discovery process at preclinical stages, as is already happening for other diseases, by reducing through rapid computational screening the time required for hit identification and lead optimization, as well as for accurate predictions of pharmacokinetics, pharmacodynamics, and toxicity [134,135].

An area where exciting progress may be expected is the targeting of disordered proteins by small molecules. Lessons learned from the thermodynamic approaches described here could have a major impact in drug discovery programs for many major human conditions where disordered proteins play central roles related to the dysregulation of their native states [29,67–69].

Another direction where major advances are likely to emerge in the coming decade is the extension of the type of drug discovery described in this review to protein condensation diseases, where a dysregulation of the balance between the native, droplet, and amyloid state appears to underlie many pathological processes [85,119,120].

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Declaration of interest

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