Mechanisms and pathology of protein misfolding and aggregation

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Abstract

Despite significant advancements in machine learning-based protein structure prediction, we are still far from fully understanding how proteins fold into their native conformation. The conventional notion that polypeptides fold spontaneously to their biologically active states has gradually been replaced by a more intricate reality in which cellular protein folding often requires context-dependent guidance from molecular chaperones in order to avoid misfolding. Misfolded proteins can aggregate into larger structures, such as amyloid fibrils, which perpetuate the misfolding process, creating a self-reinforcing cascade. The recent surge in amyloid fibril structures has deepened our comprehension of how a single polypeptide sequence can exhibit multiple amyloid conformations, known as polymorphism. The assembly of these polymorphs is not a random process but is influenced by the specific conditions and tissues in which they originate. This observation suggests that, similar to the folding of native proteins, the kinetics of pathological amyloid assembly is modulated by interactions specific to cells and tissues. Here, we review the current understanding of how intrinsic protein conformational propensities are modulated by physiological and pathological interactions in the cell to shape protein misfolding and aggregation pathology.

Introduction

Proteins execute a dazzling diversity of functionalities in cells, including roles in catalysis, structure, interactions and signal transduction. To achieve this array of functions, proteins fold into specific enabling structures or ensembles, for example, to bring together a stereospecific catalytic site or to achieve structural compatibility with binding partners. In the cell, protein folding fidelity is safeguarded by the protein homeostasis network, which includes molecular chaperones and degradation pathways. During aging, the capacity to maintain protein homeostasis declines[1,2]; a diverse group of human diseases share an underlying failure of protein folding. The consequence of misfolding can be limited to the loss of function of the misfolded protein, as is the case in cystic fibrosis[3,4]. However, misfolded polypeptides can clump together to form larger assemblies, called aggregates. These aggregates can trigger cellular stress, leading to dysfunction and eventually death, that is, they mediate a toxic gain of function.

Amyloid fibrils are ordered, insoluble protein aggregates that have a direct role in many pathologies, each characterized by the proteins deposited and the affected tissues[4,5]. They underlie many neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and frontotemporal dementia (FTD)[4,5]. Amyloids can also affect other tissues, for instance in the case of type II diabetes mellitus, or cause systemic forms of disease, such as in the case of immunoglobulin light chain amyloidosis or transthyretin (ATTR) amyloidosis[6] (Supplementary Table 1). The transfer of amyloid fragments, typically referred to as ‘seeds’, from one organism to another is often sufficient to cause further aggregation of the normal protein in the recipient by recruitment to the growing amyloids[7,8]. The pathological relevance of this observation remains under debate for many proteins, but it has wide support in the case of the prions diseases (from proteinaceous infectious particles), which are a group of fatal, neurodegenerative diseases caused by the abnormal form of the Prion protein[9]. To add to the complexity, cells also exploit amyloid fibrils as functional entities to provide physical support, to regulate activity, and as compact states in which temporary storage of protein and peptide hormones is supported prior to release or under stress conditions[4,10,11] (see Box 1).

Methodological advances have expanded our view of the biological activity of amyloids. Time-resolved and single-molecule approaches have helped in reaching a better understanding of the kinetics and thermodynamics of amyloid formation, such as the rates and pathways of aggregation and the energetics of different amyloid conformations[12,13]. Transcriptomics and proteomics have

enabled characterization of amyloid species in complex biological matrices, enabling the study of the diversity and specificity of amyloid formation[14-16]. Information derived from high-resolution methods[17-19] is now reaching levels that support the deduction of structure–activity relationships[20,21].

In this Review, we provide an update regarding the thermodynamic and kinetic principles of amyloid formation and their relation to protein folding and misfolding. We highlight key molecular drivers and discuss their relevance to end-stage amyloid structures. We discuss mechanisms of toxicity, focusing on the effect of amyloid interactions with cellular components, and the association of aggregation with healthy aging and pathology.

Theories of protein folding and misfolding

Protein folding does not proceed by random conformational sampling[22]. Instead protein sequences have been shaped by natural selection so that protein folding converges on the functional conformation[23]. Protein folding reactions are often conceptually described in terms of the so-called free-energy landscape whereby the vertical axis corresponds to the free energy of a protein, while the horizontal axis reflects the diversity of protein conformations, with similar conformations being situated in proximity to each other along the horizontal axis (Figure 1a). In its simplest form, the folding landscape exhibits a smooth funnel shape (Figure 1a-1). The broad upper portion of the funnel encompasses numerous diverse unfolded conformations characterized by high free energy. In contrast, the lowest point represents the distinctive native conformation, which possesses the lowest free energy. The energy landscape smoothly converges from top to bottom due to the progressive formation of native-like interactions, thereby increasingly favoring the native state of the protein. This situation corresponds to the original thermodynamic postulate[24] (also reffered to as Anfinsen's dogma[25]) stating that protein folding occurs spontaneously because the native conformation of a protein represents its most stable but also kinetically accessible state. (Figure 1a-1).

In reality, proteins often undergo folding processes involving intermediate states and encounter rate-limiting steps influenced by local structural propensities and hydrophobic collapse[26]. Furthermore, not all folding intermediates contribute to productive protein folding, as some can misfold by forming non-native interactions[27,28]. As a result, the energy landscapes representing

protein folding exhibit a 'rugged' rather than smooth appearance (Figure 1a-2). Protein misfolding also causes proteins to stray away from their original native folding trajectory. When misfolded proteins interact this eventually leads to aggregation. On protein energy landscapes this is illustrated by the presence of a 'dark side' where proteins become trapped in energy minima associated to aggregated protein conformations (Figure 1a-3). Protein folding reactions are therefore much more complex than initially anticipated by the thermodynamic postulate. Protein energy landscape theory revealed that the fundamental reason underlying the kinetic complexity of protein folding stems from structural frustration. This concept suggests that the local structural propensities of protein chains may not always align with, and sometimes even oppose the native conformation resulting in a rugged energy landscape with multiple local minima affecting the efficiency of protein folding. Structural frustration is probably unavoidable due to non-aligning selective pressures for function, folding, and expression levels among others. As a result, molecular chaperones[29] play a vital role in guiding proteins through the folding landscape, facilitating the avoidance or resolution of unproductive conformations, thereby achieving productive folding[30] (Figure 1a-4). An additional challenge to the original thermodynamic postulate results from protein supersaturation. Supersaturation occurs when a solute (here a protein) stays in solution for an extended period at a concentration exceeding its solubility limit at equilibrium. It has been argued that the physiological expression levels of proteins are often close to, and sometimes supersede, their solubility limit[31]. In this view, under physiological conditions the aggregated state of many proteins is thermodynamically more favorable than the native state[32] (Figure 1a-5). Supersaturation is proposed to give rise to a metastable sub-proteome, which becomes particulary susceptible to aggregation during ageing[33] and disease[34-36] (see Box 2). Finally, proteins starts to fold and assemble while still being translated on the ribosome. Co-translational folding affects the kinetics of the process, since conformational information is added as the polypeptide chain elongates, and therefore, requires chaperones for proper folding[37-39]. Thus, although protein folding is evolutionarily shaped by thermodynamic principles, the kinetic complexities arising from structural frustration and the reliance on cellular machinery often challenge the thermodynamic hypothesis[38]. This point has been illustrated in detail in a study of the folding pathway of tubulin, which interacts with the chaperones prefoldin and TRiC [40]. In contrast to the GroEL/ES chaperone (whose inner chamber primarily provides a secluded environment for proteins to fold in[41,42], called an Anfinsen

cage[43]), the folding trajectory of tubulin is shaped by guiding cross-interactions with the TriC chamber, to the extent that it cannot fold independently of TriC[40].

Overall, the presence of structural frustration, supersaturation, co-translational folding, and the reliance on chaperones[44] highlight the crucial role of kinetic control in cellular protein folding. As will be discussed later in this Review, this interplay of kinetic effects by pathological interactions and structural frustration in the amyloid conformation is also likely involved in amyloid polymorphism, again resulting in a rugged energy landscape (Figure 1a-6).

Links between conformational stability and aggregation

Protein architectures can be categorized in four main classes: globular soluble proteins, fibrous proteins, transmembrane proteins, and intrinsically disordered proteins (IDPs). Globular proteins are stabilized by amphipathic α-helices and β-sheets that assemble to form a hydrophobic core. Often these secondary structural elements are fully buried, which is why most primary sequences harbor short hydrophobic segments (6 to 10 residues). These segments are essential for the stability of the hydrophobic core (Figure 1b); however, they also constitute aggregation-prone regions (APRs) that favor intermolecular β-sheet self-assembly into amyloid-like aggregates[4] (Figure 1c). APRs are found widely across proteins, regardless of their structural class [18,45-47] (Supplementary Table 1) and can be predicted computationally (Box 3): on average, ~20% of residues in globular domains reside within APRs.

APRs are prevalent in transmembrane proteins (Figure 1b), due to the high hydrophobicity of their transmembrane-spanning domain. Conversely, given the absence of cooperative folding stems from their lack of sufficient hydrophobic amino acids, the number of APRs in IDPs is substantially lower[48]. However, many IDPs still require a degree of local structure propensity[49], often at interaction sites requiring some degree of hydrophobicity, and hence harbor APRs[50-52] (Figure 1b). Owing to their increased exposure, APRs in disordered regions of proteins are particularly susceptible to self-assembly[53], which probably explains why several proteins at the center of major amyloidosis diseases, such as the amyloid-beta peptide (Aβ) and tau (for Alzheimer's disease) and alpha-synuclein (αS; for Parkinson's disease), are intrinsically disordered (Supplementary Table 1). Molecular dynamics simulations indicate that the conformational conversion of IDPs towards an organized structure through aggregation or upon binding to functional partners follows a rugged

energy landscape that is not dictated by a global minimum, such as in the case of folded proteins, and makes them prone to parallel oligomerization pathways and amyloid formation[54]. Interconversion from an inert intrinsically disordered monomer to an aggregate-compatible compact monomer state has been proposed to occur for multiple IDPs[55-57], whereas others have proposed that lipid interactions with disordered monomers can progress to pathology[58]. Similar interactions have been suggested to lead to membrane disruption induced by amyloid-like aggregates formed by IDPs[59]. This rapid interconversion and compaction of IDPs that can lead to non-native protein interactions and aggregation might be countered by interactions with solvent molecules, counter-intuitively retaining IDPs largely in an expanded conformation in aqueous solutions[60]. Finally, transient misfolding events occur frequently between tandem repeats of neighboring domain copies incorporated in fibrous proteins[61]. In fact, neighboring domains sharing high sequence similarity are more prone to amorphous aggregation[61,62]. This is because they can expose identical APRs in proximity that increase their susceptibility to aggregate, exchange identical segments between domains, or form self-recognition contacts that introduce frustration in their energy landscape and entropically favour misfolding compared to native folding[63].

The determining role of APRs for protein aggregation has been recapitulated by many studies, starting with mutational analysis[46,64,65] and grafting experiments, in which cloning of an APR derived from an aggregation-prone protein into another, typically non-amyloidogenic sequence, is enough to induce amyloid formation[66,67]. Several studies have demonstrated their ability to assemble in isolation[45], as well as their ability to induce aggregation of the full-length protein when added as peptides in cis formation[68-71]. As such, APRs probably constitute the most common occurrence of structural frustration in proteins whereby local propensity for amyloid-like aggregation competes with and opposes global native structure.

Despite being a result of protein architecture, the intrinsic tendency of proteins to aggregate, particularly through aggregation-prone regions, poses challenges to cellular proteostasis and contributes to disease. Why has aggregation then not been eliminated by natural selection? One reason is that mutations that destabilize the native state of proteins often lead to increased aggregation propensity[72]. Proteins from extremophiles provide an illustrative example of the seemingly paradoxical nature of APRs. While exhibiting higher resistance to thermal denaturation, they harbor more rather than fewer APRs due to their more hydrophobic cores[73,74]. Conversely, the

selective pressure of Hsp90 deletion on proteins of Hsp90-dependent viruses results in less aggregation-prone, yet also less stable proteins[75]. These findings demonstrate the intricate relationship between protein aggregation and stability. Moreover, native and amyloid structures are thermodynamically correlated, as mutations that destabilize the amyloid state often also destabilize the native structure[74,76]. Secondly, the universal genetic code is conservative of both protein structure and aggregation propensity, as many single base pair substitutions result in conservative substitutions that preserve protein stability[77], but also tend to conserve protein aggregation[74,78].

An alternative thought-provoking perspective suggests that amyloid propensity in present-day proteins may have its roots in ancient protein structures. Indeed, the amyloid-first hypothesis proposes that prebiotic amyloid peptides gave rise to the universe of current protein topologies[79,80]. In this view, regions with amyloid propensity persist in contemporary proteins as remnants of this primordial influence. Interestingly, amyloid peptides have demonstrated the ability to spontaneously emerge[81] and propagate[82] in prebiotic conditions, bind RNA [83], interact with lipid membranes[84] and harbor catalytic function[85].

Kinetic partitioning of the native state

The native state of most proteins is a metastable state that is protected from misfolded and aggregated states through kinetic partitioning (Figure 1a and 1c). For proper function, the rate of folding to the native state must be higher than the rate of aggregation at physiological concentrations, and the rate of unfolding should be sufficiently slow so that the native state remains stable throughout the biological lifetime of the protein. From an energy landscape perspective, the barriers between the native state and aggregated states need to be sufficiently high (Figure 1c). In fact, proteins with a short lifespan are enriched in protein deposition diseases[72] as their turnover becomes dysregulated with age due to a decline in protein degradation[86]. Kinetic partitioning is achieved by both protein intrinsic and extrinsic factors.

Extrinsic factors

The most well-known extrinsic factors are molecular chaperones[87], which favor native protein folding, prevent misfolding, mediate degradation and reverse aggregation. A full overview is beyond the scope of this Review, but we mention a few highlights. For example, the different client-binding

mechanisms of the Hsp70 family allows for the adaptable functionality of this hub chaperone, which can ATP-dependently stabilize or unfold protein structures and prevent aggregation by safeguarding both partially folded and unfolded protein chains[88]. This plasticity is further regulated by co-chaperone activity, such as with Hsp40[89]. Another example of this cooperativity is in αS, for which six divergent chaperones recognize a motif around Tyr39; notably, phosphorylation abrogates their activity, providing a potential link for this post-translational modification (PTM) in Parkinson's disease[90]. Chaperone-mediated native folding can be catalysed even under non-equilibrium conditions through ATP consumption[91].

Chaperone-mediated autophagy (CMA) is another important pathway contributing to degradation of misfolded and aggregated species[92]. For example, DJ-1 and Hsc70 mediate autophagy of αS[93] and other metastable neuronal proteins that are at risk of aggregation[94]. Tau acetylation reroutes degradation towards macroautophagy and endosomal microautophagy but also enhances tau prion-like transmission, which is considered important for the spreading of the pathology through the brain[95]. Chaperones also differentially interact with protein aggregate species formed during the aggregation process[96] to block or disassemble protein aggregates[97]. Single-particle analysis of ClpB-mediated disaggregation revealed that it is a dynamic and fast process, with substrates refolding after exiting the pore at rates of 500 residues per second[98]. However, chaperone-mediated amyloid disassembly can also backfire if amyloid fragments (seeds) rather than monomers are produced; each fragment can start a new aggregation reaction (in a process called seeding, see below), as observed in the case of Hsp70 and tau amyloid fibrils[99] or during the cooperative action of DNAJB1 and Hsp70 in disassembling αS fibrils[100].

The other external factor affecting kinetic partitioning of the native state is translation dynamics[101], in which variations in transfer RNA abundance and compatibility with the ribosome binding site lead to differential translation speeds for different codons[102,103]. Because the genetic code is redundant (that is, multiple codons code for the same amino acid), the same sequence can be translated at different rates depending on the codons[103]. Because translation is slower than protein folding by several orders of magnitude, the idea is gaining traction that the non-uniform usage of codons in organisms (bias) is an adaptation to optimize the translation rate in favor of native folding[104,105]. Evidence for this idea includes the following: one, hydrophobic cores (thus also APRs) tend to be enriched in optimal codons[106]; two, there seems to be a bias in secondary structural preference of

codon usage[107]; three, ribosomal pausing sites in interdomain regions are proposed to enable folding between domains[104]; and four, ribosomal pausing allows time for chaperone binding[108]. Codon bias is thought to be one of the reasons why non-synonymous mutations can affect protein structure and function[107,109]. Moreover, at least in yeast, ribosomal pausing becomes dysregulated with age[110]. Notably, translation dynamics probably synergizes with interactions between the nascent chain and the ribosome[111], as well as ribosome-associated chaperones, such as NAC[112].

Intrinsic factors

To promote kinetic partitioning, evolution has selectively enhanced the presence of aggregation gatekeepers [113,114], which are a distinct group of residues that counteract aggregation. Gatekeepers achieve this by employing charge repulsion (Asp, Glu, Arg, Lys) or discouraging aggregate structure (Proline). They are typically found at the N and C-terminal flanks of APRs at the first position at which the polypeptide chain emerges sufficiently from the hydrophobic core to accommodate placement of a charged residue[115]. Aggregation gatekeepers also occur in the middle of APRs in polypeptide sequences, effectively suppressing the local aggregation propensity and leaving only cryptic APRs[116], i.e. weakly aggregating APRs that have a higher content of polar and charged residues[48,52]. Cryptic APRs are more prevalent in IDPs than in globular proteins because of fewer structural constraints[48]. Gatekeeper conservation correlates with the aggregation propensity of the APR, and this conservation comes at a cost to protein stability, suggesting that aggregation gatekeepers form an additional class of functional residues[117,118]. The codon bias of aggregation gatekeepers differs from the average for the proteome; in addition, conservation of aggregation gatekeepers occurs at the codon level, not the amino acid level, suggesting a role in co-translational protein folding[117]. Negatively charged aggregation gatekeepers are the more potent suppressors of aggregation, however, their short side chains restrict their usage to the surface of globular proteins[115]. The longer positively charged gatekeepers are more easily accommodated in the hydrophobic core but they are less efficient aggregation inhibitors[115]. As a result, charged aggregation gatekeepers seem to constitute a code for chaperone interaction: several major chaperones, such as Hsp70, have a stronger affinity for APRs flanked by positively charged aggregation gatekeepers [115]. Notably, DAXX, a polyD/E protein, has been identified as a chaperone with a high affinity for positive charges[119]. .

The wider flanking regions surrounding APRs have additional modulatory effects as well[120]. The mechanism of action of MOAG4/SERF, a known modifier of proteotoxicity[121,122], falls into this category. MOAG binds to negatively charged residue clusters distal from APRs on aggregation-prone proteins and expedites aggregation into inclusions thereby limiting toxicity of the aggregation process to the cell[123]. An α-helical region preceding the aggregation-prone polyQ track of Ataxin-7 stabilizes the latter in a helical conformation and suppresses aggregation[64,124] (Supplementary Table 1). The central tau ^{306}VQIVYK^{311} aggregation motif (Supplementary Table 1) is locked in a β-hairpin-like compact structure by its N-terminal flanking region that prevents aggregation; alternative splicing or disease-related mutations alter the modulating capacity of this upstream element[125]. Disordered flanking regions can have an inhibitory effect on APRs, as shown in the case of MPS2, an amyloidogenic protein expressed by *P. falciparum*[126]. Apart from inhibiting aggregation, such regions can also act as 'entropic bristles', which are intrinsically disordered or highly-charged short sequences that increase sequence entropy and sweep out a large area through water interactions or random movements[127,128]. As such, entropic bristles increase the fragility of fibrils and tune the seeding efficiency of aggregates by generating a larger number of short prion-competent fragments[129]; they can also simply modify the resulting morphology of aggregates formed by APRs[130].

The amyloid state and polymorphism

With the establishment of structure determination methods for proteins in the amyloid state and the increasing number of available structures, we have gained insight into the remarkable polymorphism exhibited by these structures. Several protein sequences have been observed to adopt multiple distinct amyloid structures[17,131,132]. The origins and implications of this polymorphism are subject of debate but it likely arises from the fact that these structures are not products of evolutionary selection. Consequently, numerous similar energy minima coexist within the amyloid energy folding landscape (Figure 1a-6). A compelling argument supporting this notion is that functional amyloids which have evolved toward the amyloid state, are largely monomorphic even under non-physiological conditions [133].

The ultrastructure of protein aggregates

The ultrastructure of protein aggregates varies considerably, ranging from cases involving the self-assembly of native-like folds[134] or 3D-domain swapped models[135], to primarily α-helical[136] or (most often) enriched in β-strands. The latter also form a spectrum of morphologies ranging between amorphous inclusions[137], curly fibrils[130,138], and highly ordered amyloid structures. What differentiates the highly ordered amyloid structure from less ordered forms of β-aggregation is unclear, given the latter still evades structural determination. Amyloid deposits in cells and tissues consist of long non-branched fibrous assemblies of thousands of peptides with a width of about 6-12 nm and lengths of up to several μm. Amyloid fibrils have a rope-like architecture composed of one or several protofilaments intertwined laterally along the fibril axis. The ultrastructure of amyloid fibrils often adopts a regular helical twist, but many also display a more planar ribbon-like structure. Successive advances in X-ray diffraction[139], crystallography[45], solid-state nuclear-magnetic resonance (ssNMR)[140,141] and cryo-electron microscopy (cryo-EM)[142] have revolutionized structure acquisition and vastly improved resolution. Together these efforts have greatly advanced our understanding of the amyloid fold, resulting in an explosion in high-resolution structures[19]. These atomic resolution structures confirm that amyloids form their own class of protein assemblies sharing a distinct 'cross-β' architecture[139,143] that is not observed in other protein architectures[4,5].

We now have unprecedented detail on the structure of protofibrils and how they assemble into amyloid fibrils, thereby unveiling the structural origin of polymorphism. Importantly, structures of patient-derived fibrils have revealed the association of specific polymorphs to particular pathologies[142]. $A\beta_{42}$ peptide forms two distinct polymorphs associated with familial and sporadic Alzheimer's disease[144] (Figure 2), whereas tau forms ultrastructural polymorphs that are classified between different tauopathies and share several different protofilament folds[145-147]. αS forms distinct fibrils in multiple system atrophy and Parkinson's disease[148,149]. Notably, these structures are not representative of αS fibrils formed in vitro, which differ even further when incorporating disease mutations and PTMs[150,151]. Polymorphism has also been confirmed for TDP-43[152] and PrP[153], as well as in localized[154,155] and systemic forms of amyloidosis, including ATTR[156], serum amyloid A amyloidosis[157] and light chain amyloidosis[158]. Conformational variants of various amyloids have been shown to stably self-propagate in cellular systems via the recruitment of soluble protein to the aggregates, and are characterized by diverse progression rates and distribution patterns[159-162]. As a

result, they are often referred to as prion-like strains and are invariably associated with different diseases, thus enabling accurate hierarchical classification of pathologies based on fibril structure[131]. Although amyloid structure seems to be stable across patients sharing the same pathology, it is also clear that amyloids are extremely sensitive to environmental conditions. For example, distinct strains formed by Aβ form at later stages of Down syndrome, suggesting that the convergence of aging and disease-specific factors can dictate the formation of characteristic amyloid strains[163]. Cryo-EM studies of *in vitro*-prepared amyloid fibrils of tau and αS have shown that additives such as heparin or nucleic acids can substantially alter amyloid structure[164-166], in a similar lateral mode of binding shown for amyloid-specific dyes[167]. Faithful *in vitro* propagation of *ex vivo* polymorphs is challenging: although serum amyloid A patient-derived polymorphs seem to seed accurately *in vitro*[168] this is not the case for αS[169,170]. Finally, *in vitro* assembly of tau polymorphs resembling disease-specific polymorphs is possible but highly dependent on buffer conditions, tau construct lengths and conditions of incubation, such as shaking[171,172]. The contrast between seemingly conserved disease polymorphs in patients and the variability of in vitro amyloids suggests that the pathological conditions under which disease polymorphs emerge also reflect very specific pathophysiological contexts. Factors influencing polymorphic bias include disease-associated mutations[161] and tissue- or cell-specific proteolysis[173]. Notably, PTMs also have a significant role in this process. For instance, charge neutralization by acetylation of lysine chains in tau and phosphorylation (Tyr39) of αS favour β-stacking interactions[150,174]. Additionally, ubiquitin incorporation has been proposed to alter protofilament packing in tau polymorphs in corticobasal degeneration and Alzheimer's disease[174]. Patient-derived amyloid structures are often associated with undefined non-proteinaceous electron densities filling internal cavities or decorating surfaces of unknown origin, although they are suspected to be lipids or polyions of some sort[142].

Structural determinants of amyloid stability

With hundreds of atomic resolution amyloid structures available, we can now identify general trends and properties determining amyloid structure stability[18,20]. One observation is that functional amyloids are generally associated with lower stability than disease-related fibrils (Box 1). Another immediate observation is that amyloid core structures do not always consist of full-length proteins[171,175] but rather protein fragments. This observation suggests that large segments of protein

sequences are not compatible with the amyloid state. Non-core segments constitute the fuzzy coat[53,145] or are removed altogether by proteolysis. Removal of non-core segments seems to enhance amyloid stability, probably by exposing amyloidogenic fragments and removing incompatible ones[176].

Amyloid core structures are not uniformly stable upon inspection. Roughly one-third of residues structurally support the cross-β conformation, while another third is frustrated due to unfavorable dihedral angles, main chain H-bond geometries, or suboptimal sidechain packing, with the final third minimally contributing to the amyloid fold. Structural frustration is mostly segmental and interspersed with segments forming regular β-strands. Thermodynamic analysis using empirical force fields such as Rosetta[18,21] or FoldX[20] confirm these structural observations, indicating that structurally satisfied segments also form stabilizing tertiary side chain interactions within the amyloid protofibril (Figure 3). Comparing different polymorphs of tau, αS or Aβ shows that the same segments are structurally stabilizing in different protofibrillar folds forming very similar β-sheets. Not surprisingly, many of these correspond to APRs that have long been known to be crucial for amyloid assembly, such as PHF6 in tau (^{306}VQIVYK^{311}) or ^{16}KLVFFA[21] in Aβ (Figure 3a-c and Supplementary Table 1).

Protofibrillar polymorphs, therefore, share a common framework of cross-β-favouring segments that are stabilized by alternative tertiary side chain interactions. In the case of tau, stabilizing tertiary interactions are often provided by different framework segments that either pack against each other or at the same time also serve as anchor points providing stabilizing interactions to structurally variable segments enabling these to be integrated in the cross-β core structure (Figure 3d)[20,21,177]. The same modularity is also observed in other amyloids including Aβ or αS. Together these observations depict a highly frustrated and rugged polymorphic conformational landscape[132]. Unlike native protein folding, kinetic control of this energetic landscape has not been shaped by natural selection. Instead, the kinetics of amyloid assembly are directed by pathological interactions resulting in disease-specific polymorphic bias.

Kinetic principles of amyloid formation

Amyloid formation is a complex kinetic process that involves interconversions between aggregated species in an evolving reaction mixture that gradually enriches end-stage amyloids (Figure 4a-c).

Several studies have determined the microscopic rates characterizing the key steps in amyloid assembly, including primary and secondary nucleation, elongation, fragmentation, oligomer disassembly, and monomer dissociation (Figure 4d). Nucleation, fibril elongation and fragmentation all contribute to the growth of mature amyloid fibrils and are counteracted by oligomer disassembly and monomer dissociation[178].

Nucleation

Primary nucleation, i.e. the generation of new amyloid species in a pure solution of monomers is entropically unfavourable resulting in slow and stochastic growth rates[179]. It involves the self-assembly of monomers into productive nuclei (primary nucleation, Figure 4d). Macroscopically this kinetic step results in an initial lag phase representing the time required for the nuclei to form and grow to levels at which aggregation is detectable through conventional methods (Figure 4a). This process can be accelerated by heterogeneous interactions (e.g. with lipids[180]) that increase the local concentration of monomers and significantly speed up monomer conversion. Liquid-liquid phase separation (LLPS), has also been suggested to favour amyloid nucleation. LLPS involves the formation of liquid droplets again facilitating nucleation by increasing local protein concentrations.

Secondary nucleation is differentiated from primary nucleation, by the fact that it occurs on the lateral surfaces of already formed fibrils and is catalysed by these surfaces (Figure 4d). As lateral surfaces increase with fibril growth secondary nucleation is thereby favoured, further accelerating aggregation. The difference in specificity between primary and secondary nucleation is still a matter of debate[181]. However, taken together data from various studies suggest that secondary nucleation is a less specific process. For instance, proteome analysis of surface-induced co-aggregation in bodily fluids revealed that the composition of extracted deposits did not differ substantially. This was observed despite the bodily fluids being exposed to fibrils with diverse morphologies derived from different proteins[182]. Human prolactin and galanin, two proteins that colocalise in the same secretory granules yet are dissimilar in terms of the primary sequence, form functional co-assemblies through secondary nucleation, countering the notion that secondary nucleation is necessarily sequence-specific[183]. Similarly, S100A9 co-aggregates by coating and reducing the availability of the same secondary nucleation surfaces that catalyze $A\beta_{42}$ self-nucleation[184]. Positive evidence supporting the specificity of secondary nucleation is mostly derived from research on Aβ assembly.

For instance, evidence shows that surface catalysis is both selective between $A\beta_{40}$ and $A\beta_{42}$[185] as well as enantio-specific[186]. However, another study indicated that Aβ auto-catalysis occurs independently of surface properties, suggesting that the specificity of secondary nucleation for this peptide is driven by the intrinsic propensity of the free monomer to adopt a similar amyloid fold under given conditions[187].

Conversely, primary nucleation appears to be highly sensitive to sequence variation. Thermodynamic analysis of the elongation of aggregation cores through axial addition of monomers revealed that even a single residue variance accounts for over 50% of the resulting sequences being structurally incompatible with cross-interactions[188]. Primary Aβ nucleation is also sensitive to pH variation that alters net charges, whereas secondary nucleation is unaffected[189]. Furthermore, intramolecular interactions play a crucial role during primary nucleation in facilitating the formation of initial nuclei by arranging monomers into a compact and nucleation-compatible conformation[55-57].

The morphological fidelity of secondary nucleation has also been debated. Structural evidence revealed that αS secondary nucleation is promoted by transient electrostatic interactions that involve the flexible ends of the protein excluded from the aggregation core and that these interactions promote the unfolding and local concentration of monomers, suggesting that the structure of the pre-formed core is not imprinted on the second generation aggregates[190]. In fact, amyloid strains formed through secondary nucleation have been suggested to be defined primarily by solution conditions rather than the structure of the templated fibrils[187,191]. Independent studies for Aβ[192] and insulin fibrils[193] have also demonstrated that only the fibril ends faithfully template the morphology of derived aggregates. Cryo-EM studies have identified that under conditions conducive to templated structure formation, surface nucleation can still catalyze different morphologies, indicating that secondary nucleation contributes to the increased variability in the formation of amyloid fibrils[155]. However, observations of single Aβ fibrils using total internal reflection fluorescence (TIRF) microscopy revealed have that under the same conditions, secondary nucleation typically results in the formation of fibrils that resemble the parent fibril population[13].

Elongation

Single-molecule observations have shown that fibril elongation for various amyloidogenic proteins (both functional and pathological) occurs through a stop-and-go mechanism[194,195]. This process is defined by growth bursts of constant rates with intermittent stationary periods[196]. Importantly, amyloid elongation might be polar, suggesting that fibril tips are not equally reactive in the recruitment of monomeric proteins to the growing amyloid fibril[195,197]. This unequal reactivity has also been attributed to imperfections occurring at fibril tips, reminiscent of a 'lock–dock' mechanism for elongation[198] in which the addition of monomers at fibril tips requires significant structural rearrangement of the newly added monomer[199] as well as of the molecules directly in contact at the fibril tip[200]. Monomer dissociation from fibril tips has also been observed to occur at rates that supersede incorporation of monomers; however, this balance is dependent on the relative monomer concentration[201].

Fragmentation and dissociation

Fragmentation (Figure 4d) is strongly dependent on environmental conditions, such as temperature and mechanical pertubations[202], but also relies on the intrinsic propensities of protein sequences and the morphological properties of the fibrils[203]. Fibril stability and length distributions are important determinants of fibrillar brittleness[204]. Fragmentation has also been linked to amyloid toxicity, as it influences cellular uptake of amyloid fragments generated through mechanical fragmentation[205]. This process is associated with the average size of aggregates, which exhibits an inverse correlation with the cellular uptake of amyloids[206]. Fragmentation is also associated with the generation of reverse oligomeric species and is suppressed when a critical fibril mass is reached[207]. These species are usually 'on-pathway' as they resemble the parent fibril structures and can deviate in terms of function compared to oligomers formed directly from monomer nucleation. Reverse-generated fibrils derived from mechanical fragmentation of Aβ amyloid fibrils interact with a central APR of the monomeric form similar to end-state fibrils, whereas oligomers formed during the nucleation process preferentially interacted with the C-terminal end[208]. On the other hand, oligomers formed during the nucleation of monomers are largely nonfibrillar and predominantly dissociated to replenish the monomeric pool instead of converting to mature fibrils[209,210]. The formation of such off-pathway oligomeric species is proposed to compromise the fidelity of aggregation kinetics by increasing the variability of lag phases and the amplitude of end-state

phases[211]. Early metastable oligomers inhibit nucleation and growth of fibrils by competing for monomers, generating biphasic assembly kinetics that transition to standard sigmoidal growth over a critical monomer concentration that hinders their formation[212]. Furthermore, these metastable species block secondary nucleation by attaching to fibril surfaces[213]. Off-pathway oligomers have been linked to higher levels of disorder that create repulsions or introduce entropic costs for the formation of ordered fibrils[214].

Interactions and toxicity of amyloids

The mechanisms through which amyloids exert toxicity have been a topic of ongoing debate in the field. Multiple lines of evidence suggest that small soluble species are responsible for the observed toxicity. These species have been associated with numerous detrimental effects, such as permeabilization of cellular membranes, impairment of degradation pathways, disruption of synaptic signalling and mitochondrial dysfunction[215-217]. The toxicity induced by fibrillar aggregation is attributed to mechanical perturbations, sequestration of cellular factors, and the subsequent activation of inflammatory responses[218]. Both lateral (Figure 5a) and axial (Figure 5b) surfaces of amyloids can engage in interactions with other biomolecules, including charged molecules such as polyphosphates and polysaccharides, as well as with lipids and other proteins (Figure 5b-c).

While the precise identity of many toxic species and their interfaces remains to be fully elucidated, the importance of heterotypic interactions in shaping the properties and infectivity of amyloids or promoting their toxic phenotype (see next section) is now firmly established. A prominent example includes the cross-interaction between medin and Aβ, which initiates the vascular deposition of the latter[219]. Synergistic interactions between different disease-associated proteins (including tau, αS and Aβ) typically exacerbate neuropathology [220,221]. For instance, co-aggregation of β2-microglobulin with Aβ promotes cognitive decline in Alzheimer's disease[222]. Tau aggregates originating from PS19 mice provide a compelling illustration of how heterotypic interactions can exert toxicity through various pathways. In reporter cells, PS19-derived tau seeds impair chaperone activity[223], whereas, in mouse models of tauopathy they inactivate histone demethylase LSD1 through sequestration (thus linking tau aggregation to downstream neuro-dysfunction pathways)[224]. A study of αS pathological polymorphs derived from either glial cytoplasmic deposits or Lewy bodies revealed that they are shaped by intracellular milieus and in turn associate with different toxic effects[225].

Finally, a little considered aspect is that amyloid fibril surfaces can have catalytic properties[85,226,227] (see Box 4), which might have biological consequences; for example, fibrils of Aβ catalyze the hydrolysis of neurotransmitters, such as dopamine and adrenaline[228].

Amyloids engage in diverse interactions

The fibril tips of amyloids are formed by partially buried hydrophobic residues of the top rungs[18]. Perhaps unsurprisingly, the growing ends of amyloids have been identified as the primary cross-interface with lipid vesicles[229,230] (Figure 5c). An comprehensive analysis has revealed similar findings for heterotypic protein interactions[188] (Figure 5c). In particular, the findings suggest that sequence similarity plays a crucial role for the cross-reactivity of amyloid fibil tips. Registered stacking of identical side chain residues on top of each other along the fibrillar axis enables sequence-dependent templated binding, resembling the self-interactions formed during fibril elongation[188]. Importantly, design strategies employing these properties have been used in several studies to develop novel therapeutics that target amyloids through structure-based inhibitor designs[154,188,231-234]. In these designs, the inhibitor peptides are constructed based on the APR sequence to enable binding to the fibril tip. However, these inhibitors also incorporate sequence variants in one or a few positions that block further fibril growth. Inhibitors initially developed to target an APRs of Aβ assembly were found to also inhibit islet amyloid peptide (IAPP) growth due to the sequence similarity of their respective APR sequences[235]. This observation underscores the sequence specificity of APR cross-interactions but also their ability to tolerate mismatches. Similar rules of specificity seem to support amyloid co-assemblies. Solved structures of heterocomplexes such as Aβ40 with Aβ42 or the necrosome complex (which is a functional amyloid hetero-assembly composed of successive rungs of RIPK1 and RIPK3; see Box 1 and Supplementary Table 1), have validated the necessity of sequence and structural compatibility along the fibril axis[236,237]. It is worth noting that such interactions actively steer amyloid polymorphism by altering the morphology of fibrils formed by self-assembly[188,208].

Due to identical side chain stacking the lateral surface of amyloid fibrils form regular steric patterns along the axis that are either hydrophobic or polar/ionic in nature, thus enabling repeated binding of diverse molecules along the fibril axis (e.g. amyloid reporting dyes[84,238]) (Figure 5a). As a consequence of this lateral periodicity, unidentified electron densities are often found decorating the perimeter of amyloid structures (defined by cryo-EM), suggesting the presence of repetitively

bound co-factors[239]. These densities often neighbour stacks of same-charge side-chains suggesting charge compensating polyionic co-factors binding[239,240]. Lateral surface templating has been associated with co-aggregation events involving several different proteins that do not share sequence or structural architecture[184,213,232,241]. Co-factor binding therefore likely contributes to steering amyloid polymorphism in different pathological contexts (Figure 5d).

The dynamic and structurally disordered regions surrounding the rigid cores of amyloids are referred to as "fuzzy coat regions". As a result of their increased surface accessibility and structural dynamics, these sequences are believed to modulate various amyloid activities, such as spreading, liquid–liquid phase transition and cross-interplay with other cellular components (Figure 5e)[242,243]; for example, the functional oligomerisation of Orb2 enhances translation[244]. Similarly, these regions can modulate amyloid assembly by mediating chaperone binding[245], or can lead to loss-of-function events by dragging native interactors towards co-aggregation[246].

Sequestration of proteins

Protein aggregates can sequester cellular components, causing loss-of-function or mis-localization (Figure 5f). For example, TDP-43 fragments form cytoplasmic inclusions that sequester RNA-binding proteins, leading to dysregulation of mRNA maturation[247]. FUS mis-localization in the cytosol alters its RNA regulatory functions and induces DNA damage[248]. Dipeptide repeat expansions (DPRs) resulting from C9orf72 hexanucleotide repeat expansions, which pathologically assemble in FTD and ALS (Supplementary Table 1), deplete levels of functional pATM and hnRNPA3, causing breaks in double-stranded DNA[249]. Mis-localisation of the nuclear histone demethylase LSD1 to cytoplasmic neurofibrillary tangle tau inclusions in P301S mouse models promotes neuronal cell death in the hippocampus and cortex leading to associated learning and memory defects[224]. Similarly, APRs can serve as heterotypic anchors, facilitating co-assembly of functional proteins and increasing cellular susceptibility to aggregation spreading (leading to loss-of-function toxicity or to a gain-of-function phenotype)[188,208]. Several studies have shown that the heterotypic composition of condensates can synergistically trap proteins towards co-aggregation following their maturation[220,250]. Notably, however, contradictory theories propose that hetero-interactions can also work as a buffer to control protein aggregation within condensates[251].

Sequestration can also disrupt cellular proteostasis. PolyQ expansions from huntingtin or ataxin-7 sequester the HSJ1 chaperone, leading to proteostasis imbalance and aggregation of ataxin-3[252]. Tau extracts from the brains of PS19 mice impair protein folding and clathrin-mediated endocytosis by inactivating HSP70, HSP90 and J-domain chaperones, an effect that can be reversed by exogenous addition of a small molecule that replenishes cytosolic chaperone expression[223]. RNA-induced tau and Aβ aggregates co-deposit ribosomal 80S, which reduces translation efficiency in yeast cells[253]. Cryo-electron tomography (cryo-ET) showed that intracellular inclusions formed by different aggregation-prone polypeptides recruit and impair the functionality of proteasome subunits (Figure 5f), along with TRiC/CCT and ribosomes, providing visible intracellular evidence of spatial isolation of proteostatic cellular components by protein aggregates[254,255]. Importantly, however, proteasome inactivation has also been proposed to occur in a non-sequestered fashion. Oligomeric species derived from αS, Aβ and Htt-53Q sharing significant structural similarities bind with high affinity and inhibit the functionality of the 20S proteasome through allosteric impairment[256]. Cryo-ET also revealed that amyloid-like fibril aggregates can cause gain-of-function toxicity resulting in lysosomal defects[257] and impair vesicular trafficking as shown in cases of Alzheimer's disease and Parkinson's disease pathology[258].

Sequestration by amyloids can also be polymorph specific. For instance, single particle analysis showed that a size-dependent population of patient-derived aggregates exerts its toxicity by penetrating cells and binding to proteasome subunits causing their inactivation[259]. Differential proteasome inhibition was also observed for two distinct αS strains prepared under different in vitro conditions[260].

Membranes and lipids

Lipid–amyloid interactions have long been recognized as a source of toxicity in proteinopathies. For instance, Lewy body formation, involving the incorporation of membranous organelles, has been proposed as a major contributor to toxicity in Parkinson's disease, surpassing that of αS fibrillation[261]. However, the exact impact of lipid interactions on the morphology and toxicity of aggregates is still a subject of debate. The functionality of αS in presynaptic vesicle docking is affected by the composition of biological membranes[262]. In line with this observation, pathological aggregation of αS has been proposed as an after-effect of its dysregulated binding to its natural

substrate PIP3, a process that is initiated by a loss-of-function of synaptojanin 1, a phosphoinositide phosphatase and a risk gene for early onset Parkinson's disease[263]. Similarly, apolipoproteins participate in native-like lipid interactions in a mechanism evolved to protect against the exposure of their APRs and their conformational switching from α-helical to cross-β amyloid-compatible folds[264,265]. NMR spectroscopy identified that early APR-driven interactions enable the partial insertion of Aβ into membranes, a process that promotes nucleation and shapes the morphology of the derived fibrils[266]. Similar interactions promote the segmental assembly of αS on the surface of anionic phospholipids[267]. Free lipids are proposed to shield the hydrophobicity of exposed APRs in a chaperone-like manner, but, above a critical concentration, they can also enable the insertion of oligomeric species into bilayers[59].

The molecular composition of membranes also determines their differential binding affinity to different amyloid species. For example, lipid bilayers generated by lipid oxidation either promote the nucleation of monomeric Aβ, stabilize non-toxic oligomers that are still capable of compromising the integrity of bilayers, or reform on-pathway protofibrils that exhibit clear toxicity[268]. Similarly, ApoE4, a genetic risk factor for Alzheimer's disease, is linked to lipid dysregulation in Alzheimer disease brains. It impairs cholesterol transport causing intracellular lipid deposition[269] and myelination dysfunction[270]. However, ApoE4 also accelerates Aβ deposition[271] and hinders its clearance[272]; therefore its exact role in Alzheimer's disease remains unclear.

Lipid–oligomer interactions are in the spotlight for understanding amyloid toxicity. Cryo-EM determination of oligomers formed by Aβ when fused to an α-hemolysin toxin revealed that it forms a lipid-soluble heptameric pore complex[273], reminiscent of the formation of similar pores by other amyloid-forming proteins[274,275]. Lipid–oligomer interactions have also been labeled as highly dynamic in several systems. Solution and solid-state NMR have revealed that two different species of αS oligomers are largely disordered or require only local folded elements when disrupting membrane integrity[276]. Soluble oligomeric Aβ species formed at different maturation stages induce membrane permeabilization or an inflammatory response as a function of size[277]. Conversion of dynamic non-amyloid-like oligomeric IAPP species has been linked to gain-of-function toxicity by inducing vesicle perforation[278]. However, another study showed that IAPP-induced vesicle leakage is a biphasic process mainly attributed to secondary nucleation and the gradual elongation of fibrillar seeds in the presence of membrane particles of various lipid composition[279].

Mature amyloid fibrils have been primarily linked to toxicity as inducers of mechanical stress that can cause tissue damage, which partially also includes damaged membrane components[280]. Direct structural evidence confirms the binding of lipids to mature amyloid fibril aggregates[230]. Cryo-EM revealed that αS forms distinct fibrils in the presence of lipids, which in turn were found to decorate their exposed hydrophobic patches, liaise as cross-contacts for their alternative quaternary organization, or channel through internal cavities of the amyloid core[281]. Correlative light and electron microscopy analysis of Lewy body inclusions from Parkinson's disease post-mortem human brain tissue exposed a crowded presence of lipid components[282]. Similar findings were revealed by cryo-ET in deposits formed in a cell culture reporter model of systemic AA amyloidosis[229]. Impressively, this study also identified that the primary site of interaction between amyloids and lipid vesicles corresponded to the fibril tips rather than their lateral surfaces, suggesting an amyloid-driven specificity for lipid interactions (Figure 5c)[229]. This finding might explain why lipid interaction evidence is scarce despite the numerous solved ex-vivo structures. Stringent purification protocols for aggregate extraction might promote this selective bias, although analysis of lateral amyloid surfaces indicates that fibril cavities are typically narrow and occupy polar residues, thus hindering lipid binding[84]. Indeed, efforts tracing aggregate surface hydrophobicity of αS aggregates revealed that oligomeric species expose more hydrophobic patches than their fibril counterparts[283]. Confirming the above, sub-micellar lipids were shown to catalyse apolipoprotein C-II aggregation, yet no lipids were recovered as co-factors in contact with mature fibrils. Similarly, cholesterol-containing lipid surfaces seemed to massively catalyse primary heterotypic nucleation of Aβ[284], whereas aminosterol derivatives can facilitate the secondary nucleation of Aβ and reduce its toxicity in *C. elegans* either by competing for surfaces (and thus causing the displacement of oligomers) or by promoting the formation of less toxic mature species[285].

The emerging role of microbiomes

External factors have also been associated with aggregation disorders although the exact nature of this relationship is yet to be defined. Pathogen-associated molecular patterns (PAMPs; short conserved microbial motifs that are absent in the host) from bacterial infections activate immune responses, triggering microglia activation and oxidative stress which are both downstream responses linked to neurodegeneration. Accumulation of endotoxin lipopolysaccharide (LPS)[286]

owing to systemic infection is associated with various aggregation-based pathologies[287], inducing neuroinflammation[288] and morphological changes in amyloid fibrils[289]. Interestingly, the amyloidogenic serum amyloid A protein (SAA) suppresses the effects of LPS and ameliorates toxicity in mice[290].

The gut–brain axis has a role in aggregation diseases, but this topic is still in its infancy. Both the composition of the gut microbiome and dietary changes affect Alzheimer's disease and Parkinson's disease[291,292] to the level that gut flora and dietary restructuring is considered a therapeutic strategy against disease progression[293,294]. The composition of the gut microbiome has also been linked to levels of disease biomarkers such as ApoE[295]. Microbiome-generated functional amyloids can interact with brain amyloids, influencing aggregation and causing behavioral and motor impairments[296-298]. Bacterial amyloid chaperones can inhibit pathological amyloids[299], while amyloids themselves can exhibit antimicrobial activity[300,301]. For instance, Aβ can form toxic oligomeric pores with bactericidal effects[302]; sequence similarity between Aβ and bacteriocins might have evolved as a strategy to tune an immune response against the latter[302].

The interplay between the intestinal environment and amyloids has also been exposed. For instance, intestinal defensins with antimicrobial activity cross-interact and prevent further propagation and cell cytotoxicity of Aβ, IAPP and calcitonin[303]. Microbiota-excreted fatty acids enhance Aβ plaque formation in mouse models of Alzheimer's disease[304]. In light of the ability of other metabolites to template amyloid formation[305,306], this concept raises further questions regarding the role of diet and food in amyloid pathologies[307,308]. Indeed, food proteins have been shown to form amyloid fibrils in isolation (Box 3), as well as to cross-seed, resulting in inhibition[309] or exacerbated pathological amyloid formation[310]. These findings raise concerns in terms of the safety and impact on health of common food processing approaches that rely on aggregate formation, such as gelation or foaming[311-316].

Finally, the relationship of amyloids to viruses is also gaining traction. For example, the HSV-1 viral corona promotes aggregation of Aβ both in vitro and in animals[317]. Similar cross-interactions have been validated between SARS-CoV-2 and other viral infectious agents with amyloid proteins[318]. The SARS-Cov-2 nucleocapsid protein utilizes electrostatic interactions to accelerate αS fibrillation and induce cell death[319]. In fact, parallel studies have shown that SARS-Cov-2 proteins can also form

amyloids in isolation[320,321], with similar results deriving for other viral species, such as the Nipah and Hendra viruses[322]. Viral infectivity has also been indirectly associated with neuroinflammation, protein quality control dysfunction and spreading of misfolded products[323,324]; protein aggregation has been proposed as a strategy for the development of novel types of anti-viral agents[69].

Conclusions and perspectives

Progress in treating protein misfolding diseases is evident from FDA and EMA approval of therapies such as tafamidis for patients with familial amyloid polyneuropathy[325], or migalastat, which rescues the folding of α-galactosidase in Fabry Disease. Voxelotor has shown promise as a conformational corrector of hemoglobin in sickle cell disease[326], similar to several approved correctors of the folding of the CFTR conductance channel (ivacaftor, tezacaftor and elexacaftor). Even in the challenging field of neurodegenerative diseases, clinical trials with lecanemab in Alzheimer's disease have shown promising progress. Specifically, selected patients with early mild cognitive impairment, even at later stages of the disease, experienced a moderate slowdown in the decline of cognition and function[327]. This breakthrough comes after years of uncertainty regarding the role of aggregates in Alzheimer's disease, compounded by unsuccessful clinical trials, controversial studies, and a lack of effective treatments[328,329]. Our deepened understanding of the structural characteristics, kinetics and interactions of amyloid structures has led to significant advancements in the development of engineered therapeutics. Notably, there has been an increase in structure-based approaches focused on the creation of highly potent and specific anti-amyloid biomimetics[330-332]. These designs aim to target the attachment to growing fibril ends and thus block axial propagation, effectively combating the assembly of various proteins such as tau[188,231], αS[232], Aβ[233], IAPP[154] and transthyretin (TTR)[234]. Heterotypic recognition has also been employed to raise antibodies[333,334] and to engineer custom protein designs[232] that slow down the formation and toxicity of aggregates. Similarly, our improved understanding of the factors dictating amyloid formation and stability is now being harnessed to develop synthetic functional biomaterials with diverse applications, including catalysis, supporting scaffolds, and bioproducts in the food industry (see Box 4).

These encouraging results, along with breakthroughs in protein structure prediction from AI-based approaches (Box 3) and direct structural determination of amyloids from patient tissues, are galvanizing the field and laying the groundwork for future breakthroughs. Despite this progress, our

understanding of protein aggregation is still in its infancy. Critical features, such as the spatiotemporal dynamics of early amyloid formation, the diversity of oligomers, the involvement of on and off pathway species, and the true origins of toxicity in disease, remain subjects of debate. It is increasingly evident that multiple overlapping processes and pathways contribute to protein aggregation. However, we are now reaching a point at which we can deduce the structure–function relationship of these species more accurately. These insights provide hope that the fundamental research discussed in this review will ultimately benefit patients worldwide, improving their quality of life.

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Figure Legends

Figure 1. The role of frustration and aggregation prone regions (APRs) in protein folding and aggregation. (a) Folding landscapes are roughened by structural frustration requiring kinetic control. Proteins are evolutionarily shaped by opposing requirements: protein folding requires sequences that favor native-like interactions while the opposite is often required for functional reasons (catalysis, protein dynamics, interaction and protein stability). Natural protein sequences are therefore structurally frustrated resulting in suboptimal folding kinetics that also favour alternative amyloid-like protein assemblies. The native conformational ensemble, therefore, is one of many local minima in a rugged protein conformational landscape requiring extensive kinetic control to be achieved. This rugged landscape can include multiple additional minima corresponding to aggregated states, with multiple factors working in balance to either steer towards (protein supersaturation, polymorphism and more) or away (e.g. molecular chaperones) from this side of the funnel (dark side). (b) Essential states of functional proteins in cells. Due to structural and functional requirements, ~20% of natural protein sequences are constituted of hydrophobic segments (less in intrinsically disordered proteins). Following this principle, APRs are integral factors that facilitate the folding of soluble protein domains and frequently occur in transmembrane domains or as binding surface in intrinsically disordered proteins. These segments generally adopt a regular secondary structure in native structural ensembles. (c) Negative selection and chaperones provide kinetic control over APRs. Natural selection favors native-like folding by minimizing the effect of APRs. Elements of negative design opposing aggregation include gatekeeper residues or entropic bristles. Structural frustration due to APRs is controlled by both negative selection and chaperones.

Figure 2. Structural determinants stabilize and are shared between polymorphic amyloid fibril structures. (A) Schematic representations of one cross-sectional layer of polymorphic Aβ fibril cores extracted from the brains of patients with different pathologies or post in-vitro amplification (seeding). The structure on the left (type I filaments) corresponds to the primary fold isolated from the brains of patients with sporadic AD (sAD). The structure comprising two S-shaped packed protofilaments, in the middle (type II filaments), was mostly found in the brains of patients with familial type (fAD). The latter was extracted also from several other pathologies, including patients with aging-related tau astrogliopathy, Parkinson's disease, dementia with Lewy bodies and

frontotemporal dementia. Similarly, both type I and type II filaments were recovered in minor population from fAD and sAD cases, respectively[144]. Fibrils prepared in vitro after seeding $A\beta_{40}$ with extracts from Alzheimer's disease patients resulted in the formation of filaments with a distinct amyloid core, as shown by the structure on the right. Individual residues are shown as circles and are colored-coded based on the per residue energetic contribution to overall fibril stability. Values were obtained from Stamp-DB, a public repository for the structural and thermodynamic classification of amyloid structures[19]. Despite their evident structural variability, different polymorphs share overlapping energetic frameworks composed of similar stabilizing sequence segments.

Figure 3.

Per residue cumulative energetic contributions (shown in the y-axis) of polymorphic cores of amyloid fibrils formed by (a) tau, (b) Aβ and (c) αS, clustered and colored-coded based on disease relevance (as shown in the figure legends). Circular histograms, with protein residues shown along the perimeter and highlight that polymorphic cores share overlapping energy profiles, with common stabilizing regions (color-shaded segments) that correspond primarily to previously experimentally determined APRs of each protein[47,160,177], interspersed with regions of structural frustration. Circular histograms were generated using the ggplot2 library in R. (d) A common framework contributes to the stability of tau fibrils derived from different pathologies. Aggregation-prone regions form important stacking interactions that typically 'clamp' together the termini of fibril cores or participate in energetically pivotal packing interactions that have been identified by parallel thermodynamic studies[19-21]. Cores are color-coded based on per residue energetic contribution as described previously (chronic traumatic encephalopathy, Pick's disease and progressive supranuclear palsy architectures are shown as overlayed transparent structures to highlight common interactions)[19,20]. Alzheimer's disease – AD, Cerebral amyloid angiopathy – CAA, Primary age-related tauopathy – PART, Gerstmann-Straussler-Scheinker disease - GSS, Chronic traumatic encephalopathy -CTE, Corticobasal degeneration - CBD, Argyrophilic grain disease – AGD, Pick's disease – PiD, Globular glial tauopathy – GGT, Progressive supranuclear palsy – PSP, sporadic Alzheimer's disease – sAD, familial Alzheimer's disease – fAD, Multiple system atrophy – MSA.

Figure 4. Overview of protein aggregation kinetics and processes that contribute during amyloid fibril formation. (a) Mainstay kinetics of amyloid fibril formation (blue line). Kinetics are characterized by a starting lag phase during which initial nuclei and 'on-pathway' oligomers are formed and reach aggregate concentrations that can be monitored in bulk aggregation assays. This lengthy lag phase can be surpassed by seeded experiments (shown as a red line) during which pre-formed aggregation nuclei are supplied exogenously. This process is followed by a shorter growth phase during which the rapid recruitment of monomers is catalyzed by fibril ends or by secondary nucleation on lateral surfaces that overtakes the aggregation process. Eventually, the process reaches an equilibrium that appears as a plateau phase in typical readouts. (b) Overlapping presence of different species during different phases of aggregation growth. During early phases of nucleation, it has been suggested that monomers adopt a compact aggregation conformation that can gradually lead towards the formation of oligomeric and prefibrillar species. During elongation, these structures can promote the growth of protofilaments and mature fibril aggregates that are formed through lateral interactions of the former. (c) Key species during the formation of amyloids. (d) Key kinetic processes that take place during the formation of amyloid aggregates. Templating that proceeds through primary nucleation results in slow and stochastic growth rates[179], whereas secondary nucleation becomes proportionally faster based on amyloid growth, as it occurs on the surfaces of preformed aggregate species. Aggregate growth is then promoted by further incorporation of monomers during elongation, whereas fragmentation, which can occur throughout the kinetic process, does not change the number of aggregates but can shift the balance between processes (for instance, primary and secondary nucleation) as it alters the number of reactive sites by modifying the numbers of reactive species.

Figure 5. Amyloid interactions with other biomolecules can have significant implications for aggregation-related mechanisms of toxicity. (a) Lateral surfaces of amyloid fibrils have been proposed to self-catalyse their growth through secondary nucleation, but also to facilitate cross-interactions with important co-factors such as polyanionic nucleic acids and polysaccharides. The same surface might also promote interaction with lipid bilayers. (b) Heterotypic interactions with other molecules (such as lipids) or integral sequence properties (such as post-translational modifications) can assist with shaping the morphology of derived aggregates. (c) The growing ends

of amyloid fibrils are reactive surfaces that promote heterotypic interactions with other proteins or lipid bilayers. (d) Heterotypic interactions with other co-factors can facilitate stabilization and steer the morphology of amyloid fibril structures by lowering the energy barriers of specific polymorphs. (e) Non-core components of fibrils, such as the fuzzy coat, have also been shown to promote the functionality of fibrils by harbouring interactions with other biomolecules[53]. (f) The heterogeneity of amyloid deposits in patients can be explained by several mechanisms of toxicity. The interplay of amyloids with the proteostatic machinery, including molecular chaperones, as well as the presence of a metastable subproteome that exists beyond its solubility limit and is therefore susceptible to widespread proteostatic collapse could explain the heterogeneous composition of in vivo deposits. Natural co-factors of fibrils are often co-deposited with amyloids and could lead to both a loss-of-function phenotype or gain-of-function toxicity events.

Box 1: Functional amyloids

Functional amyloids have interesting properties that can be employed in biological systems[335] (Supplementary Table 1). For example, amyloid filaments are often used as structural support or as protective layers[336-340]. During hormone secretion, amyloid aggregation acts as a temporary storage of polypeptide material prior to secretion[341,342]. Finally, amyloid aggregation is a modification of the functional state of a protein, which can be employed to encode molecular memory through faithful templating, such as in the case of the yeast prions[343], and also in terms of synaptic memory, as shown in the drosophila brain[344].

Early structural evidence indicated that functional amyloids form continuous 'cross-β'-like structures such as β-helices or β-solenoids, thus diversifying structurally from their pathological counterparts[345]. The repetitive nature of sequences involved in the formation of functional amyloids was proposed to support this conformation, with successive rungs corresponding to individual or sets of repeats that are capped at their ends[345-349]. Early NMR-derived structures of the HET-s prion validated this notion[141], similar to cryo-EM results and previous modeling work revealing that the major curli subunit (which is the primary scaffold for the formation of bacterial biofilms) adopts a canonical β-solenoid fold[350,351]. However, more recent structural findings have shown that functional amyloid scaffolds are much more complex than previously considered. This complexity is particularly

evident for short sequence segments. For example, the necrosome complex is composed of heterotypic amyloid fibrils that are formed by the homologous RIPK1 and RIPK3 proteins and contain an unusual ladder of alternating serines and cysteines[236]. Antimicrobial sequences expressed by amphibians form fibrils that can conformationally switch from a typical cross-β fold to an α-helical fibril arrangement when found in the presence of membranes[301].

Functional amyloids are also primarily characterized by largely hydrophilic cores in contrast to the majority of pathological amyloids[352]. The hnRNPDL ribonucleoprotein (involved in transcription and RNA-processing) forms non-toxic fibrils that bind nucleic acids[353]; their core consists of a highly hydrophilic residues incorporating several water channels. The low complexity domain of hnRNPA2 also forms fibrils with a hydrophilic core but further introduces backbone kinks that facilitate the formation of reversible hydrogels containing hnRNPA2 fibrils[354]. Orb2, a synaptic translation regulator involved in memory, forms amyloid folds with a core consisting of glutamines and histidines in a pH-sensitive mechanism, suggesting that these fibrils might also have a dynamic nature[355]. This reversible property extends to β-solenoids, as in the case of β-endorphin fibrils[356], which are stored in acidic secretory granules and retain a glutamate residue in a critical protonated state in their core; on exocytosis, the pH change promotes the release of the peptide hormone in the blood in a stepwise manner[357]. Together, these findings suggest that functional amyloids utilise atypical amyloid folds that promote the stacking of polar and charged side chains to promote their functionality and reversibility, in contrast to the rigid hydrophobic packing of their pathological counterparts[352].

Box 2: The impact of artificial intelligence

Significant advances have been made in protein structure prediction with the introduction of state-of-the-art artificial intelligence (AI)-based methods. One notable milestone was the arrival of AlphaFold, which employs established structural knowledge from the Protein Data Bank (PDB) and co-evolutionary sequence analysis derived from multiple-sequence alignments[358]. Through training a deep neural network to predict pairwise distances between residues in random sequences, AlphaFold successfully unveiled the structural folds of 98.5% of human proteins, including novel folds not previously observed[358]. The efficacy of AlphaFold was independently validated by a following collaborative study[359] and although its performance is less successful for dynamic regions, other

studies demonstrated that when combined with NMR or molecular dynamics-derived parameters, it can be utilized to predict protein dynamics and flexibility[360,361]. Moreover, when integrated with experimental data from crystallography or cryoEM, AlphaFold substantially improved protein modeling quality and coverage[362]. Additional methods, such as RoseTTAFold[363], ColabFold[364], and trRosettaX-Single[365], have emerged to enhance user accessibility, speed, and accuracy of predictions on protein folding and complex formation. There has also been a focus on expanding structure predictions to macromolecular protein complexes[366,367], although accurate predictions for asymmetric complexes remain challenging[368]. Furthermore, ongoing efforts aim to predict protein-cofactor interactions[369] and nucleic acid conformations[370]. Lastly, large language models, like ProGen[371], offer substantial benefits to the field of protein design by generating new sequences with predictable functionality, thereby showcasing the potential for further advancements.

Despite the advances made in predicting protein folding and function, the daunting task of predicting protein aggregation remains exceptionally challenging, even for AI-based methods[372]. Protein aggregation defies the influence of evolutionary pressure that shapes sequences, which is the foundation of such sequence-based methods. Additionally, the morphological adaptability of amyloids surpasses Anfinsen's dogma, which states that a particular structure is encoded by a single sequence.

The example of AlphaFold clearly demonstrates that achieving reliable prediction capabilities necessitates a much larger ensemble of structural information. Despite the remarkable progress made by cryo-EM and solid state NMR in determining the structure of fibril aggregates, we are still some distance away from attaining such levels of prediction accuracy. However, the increased availability of amyloid structures has spurred the development of computational approaches that focus on analyzing the stability and factors that govern protein aggregation[18,20,21]. In this light, machine learning has made considerable advancements in predicting the propensity for aggregation based on protein sequence[52,373,374], as well as for the breakdown of aggregation kinetics[375]. Additionally, machine learning has shown promise in the field of medical diagnosis and amyloid disease classification[376-378].

Box 3: Supersaturation

Supersaturation refers to a concentration of a solute that exceeds its solubility limit, which is defined as its concentration at equilibrium in the same given conditions. This idea extends to parts of the proteome expressed at levels beyond their solubility, a condition that sets these proteins at a high risk of aggregation and requires strict proteostatic control[35]. High local concentrations have been suggested to shift Anfinsen's postulate from favouring intramolecular interactions and natural folding towards conditions promoting intermolecular contacts and assembly[379]. Aggregation-prone proteins, for which the solubility barrier is lower, are particularly susceptible to this shift[379,380]. The environmental context also influences the levels of protein susceptibility, as conditions reducing the solubility barrier of proteins — such as membrane interactions or charge neutralisation — can promote their assembly in a salting-out-like process[56]. Regardless, supersaturation as a general mechanism has been suggested to drive massive aggregation and increased cellular vulnerability in major neurodegenerative disorders[35,381]. In fact, specific pathways enriched in supersaturated proteins are downregulated in Alzheimer disease brains to mitigate massive subproteomic collapse in conditions of compromised proteostasis[382]. Supporting this finding, single-cell transcriptomics and subcellular proteomics uncovered an enrichment of metastable proteins related to synaptic function and mitochondrial energy metabolism in protein inclusions related to neurodegeneration, thus providing a tracible pattern for selective vulnerability of neurons to aggregates[383]. Proteome analyses of the composition of inclusions derived from myopathy patients revealed a similar metastable proteome that might be linked to muscle motor dysfunction[384]. The importance of the protein quality control machinery in keeping such metastable subpopulations in line is undeniable. This premise questions, however, whether supersaturation is a driving force of aging or vice versa. Are proteins that are expressed beyond their solubility a threat during early life when protein quality control is not yet compromised? There are arguments to be made to counter this notion. For instance, mass spectrometry data in C. elegans revealed that most proteins express above their limits of solubility and that their monitoring is lost as a result of the functional decline of the machinery due to aging[385]. Gene co-expression analysis identified the endosomal–lysosomal and ubiquitin–proteasome systems as key pathways promoting clearance and preventing massive proteostatic collapse in Alzheimer disease[386]. On the other hand, it is also possible that supersaturation drives cell aging by compromising proteostasis. Impairment of certain molecular

chaperones exacerbated the widespread collapse of a metastable proteome induced when targeting the activity of ATM and DNA topoisomerases[387]. In line, loss of chaperone-mediated autophagy in mice was shown to alter neuronal function by synergistically promoting collapse of a metastable proteome and increasing cellular vulnerability to aggregation toxicity[94].

Box 4: Synthetic amyloids

Increasingly, synthetic amyloid fibrils are being explored for various applications, ranging from structural scaffolds, improved techno-functional properties in food, innovative catalysts[388], to the deliberate knock-down of target proteins by targeted aggregation.

Materials

The chemical stability, fibrillar nature and tensile strength of amyloid fibrils makes them attractive components of novel materials[389]. Moreover, amyloid fibrils can be generated from short peptides whose sequence can be rationally designed. For example, synthetic spider silk was constructed on the basis of amyloid architecture and showed tensile strength and toughness that surpassed some naturally occurring silks[390].

Catalysis

Amyloids initially appeared in catalytic applications by tethering existing enzymes to an amyloid backbone[391,392], but increasingly the surface of the amyloid itself is being employed directly for catalysis, either through the immobilization of metal ions[85,226,227], prosthetic groups or co-enzymes[393-395], or simply by the positioning of charged side chains on the amyloid surface[396]. The concept of surface-induced catalysis suggests that coordinated interaction interfaces are essential for this functional process. However, the cryo-electron microscopy structure of a catalytic amyloid revealed that these too are susceptible to polymorphy[397]; specifically, metal-binding sites were observed to form at the interface of protofilament contacts, indicating that polymorphism was instead critical in enabling the functionality of these filaments.

Food

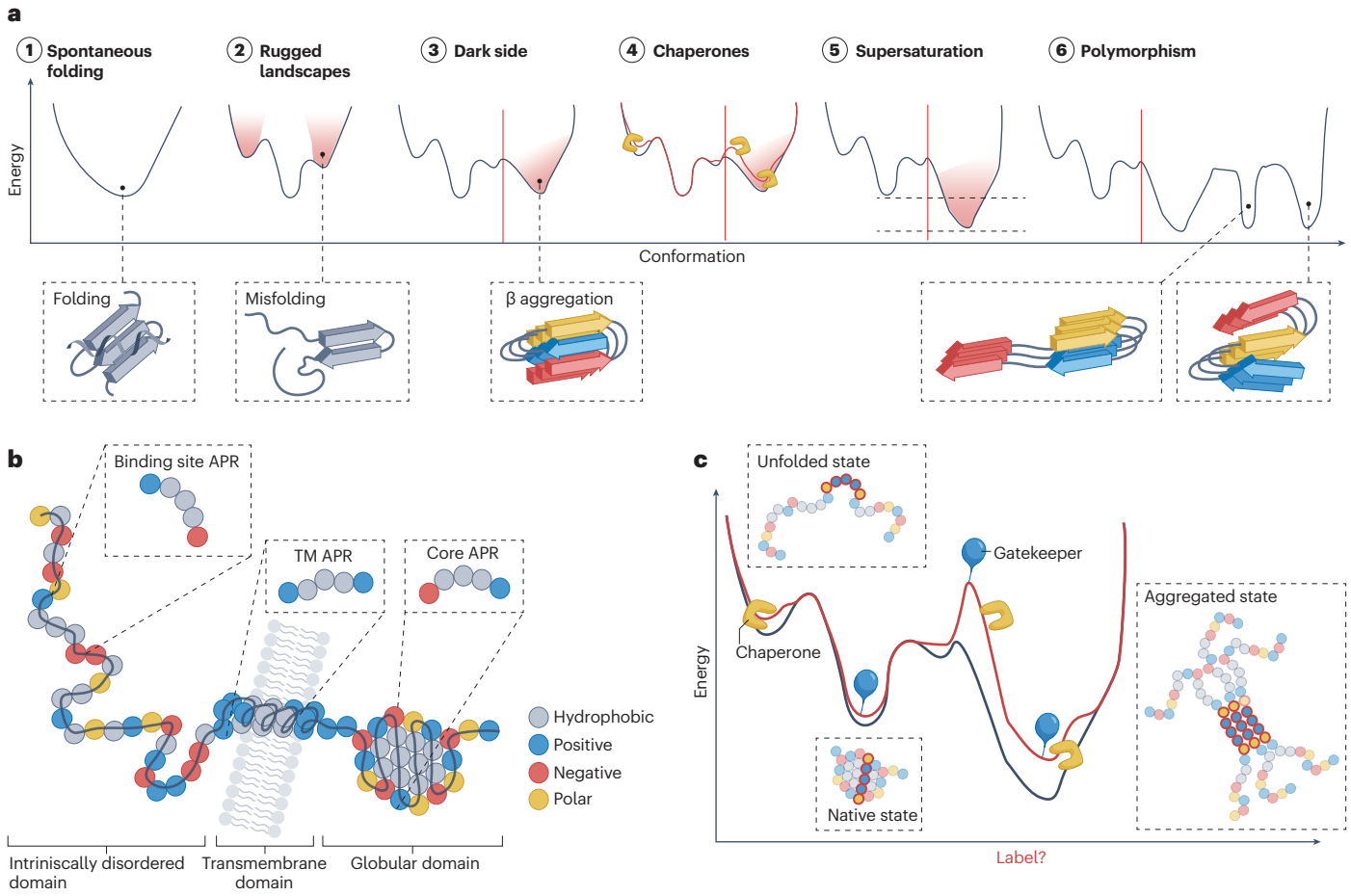
Proteins have a techno-functional role in food applications, such as gelling, foaming and emulsifying. Evidence indicates that the fibrillar state of food-born proteins can improve (some of) these properties and that common food processing techniques promote their formation[311-316]. Given that bioavailability is higher in the amyloid form (in which only the amyloid core is protected from

proteases) than for the folded protein, increasing the fibrillar protein content of certain foods might one day lead to a reduced need for protein, in particular of animal-derived protein[138,398-400], although the impact on human health requires further investigation[401].

Targeted aggregation

Our group has shown that short aggregation-prone peptides can be used to induce the aggregation of selected target proteins. This approach is based on the observation that most naturally occurring polypeptides contain aggregation prone regions that tend to be unique within their proteome, and that amyloid assembly displays sequence specificity[402]. We have shown the potential of this approach in combating bacterial strains that are resistant to established antibiotics[70,403,404], viral infections[69], and in an oncological setting[68,71]. Moreover, derivatives labelled with radionuclides have potential as PET tracers[405].

Fig 1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Core APR
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Positive
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Fig 2

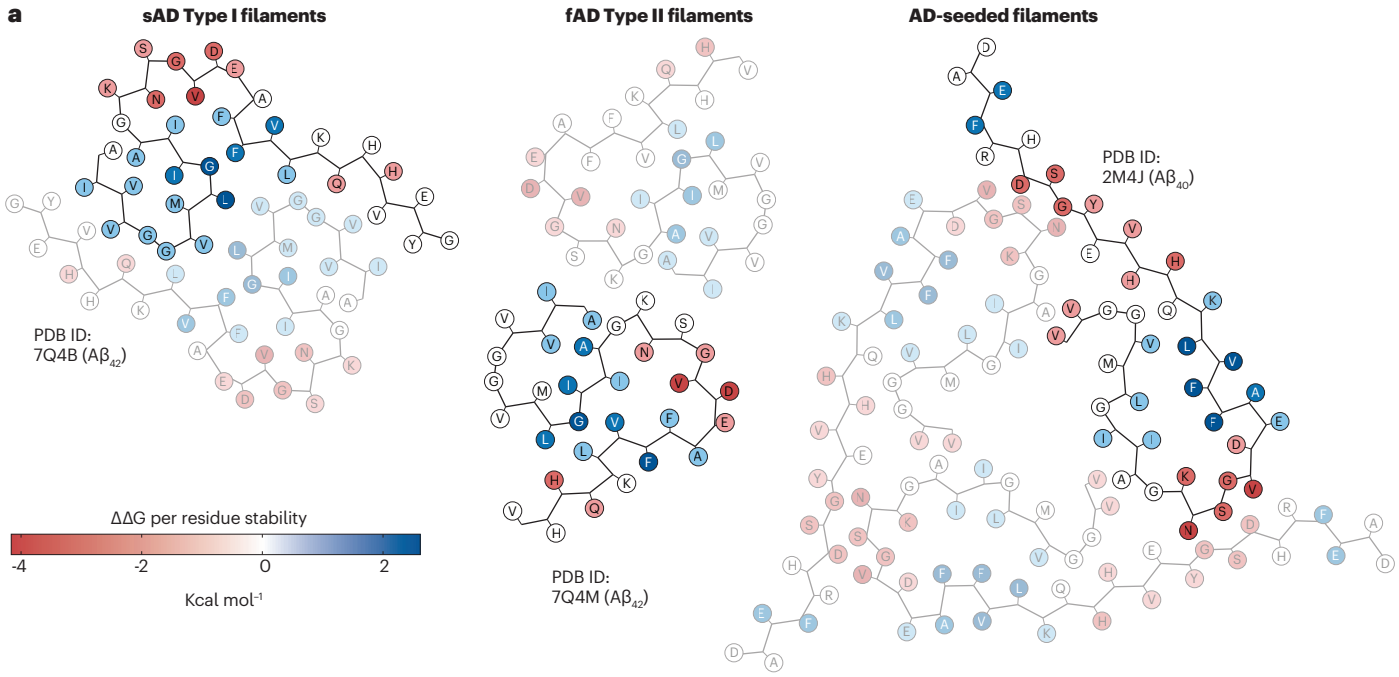
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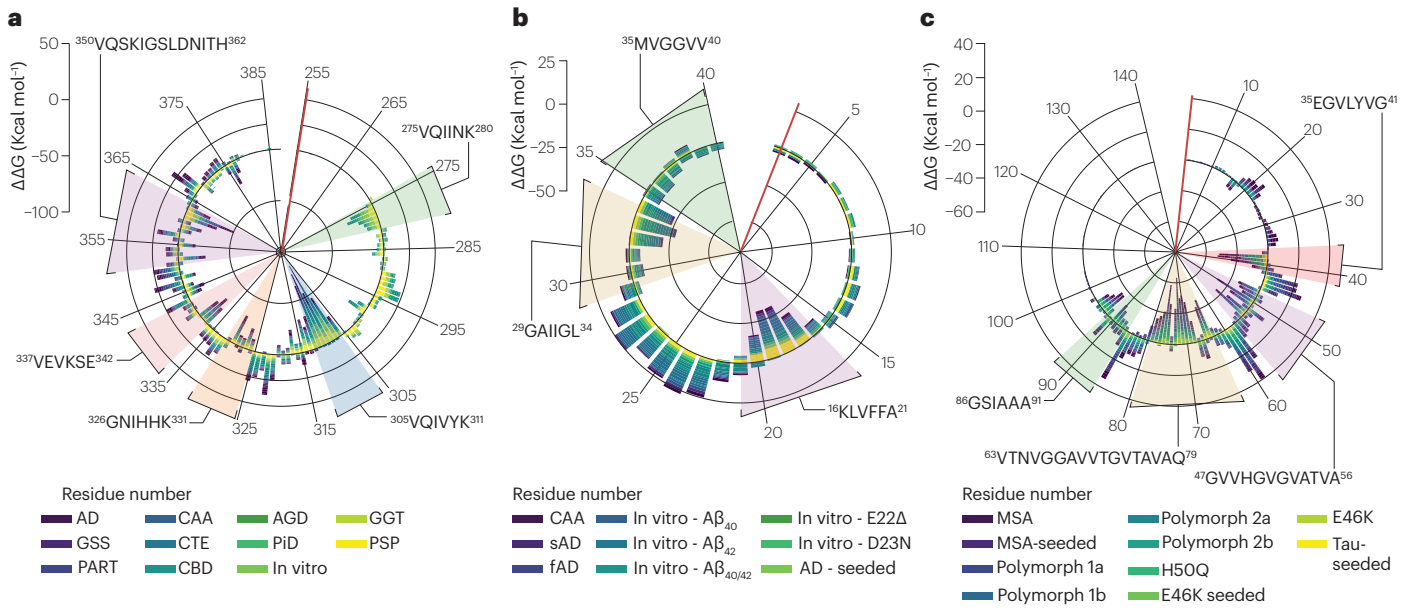
Fig 3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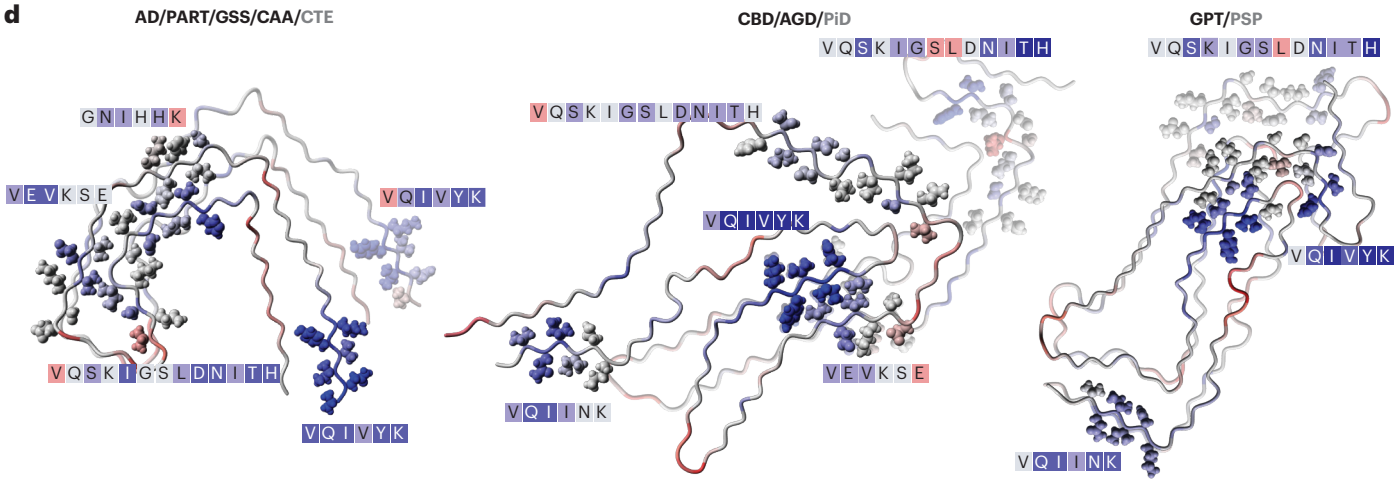
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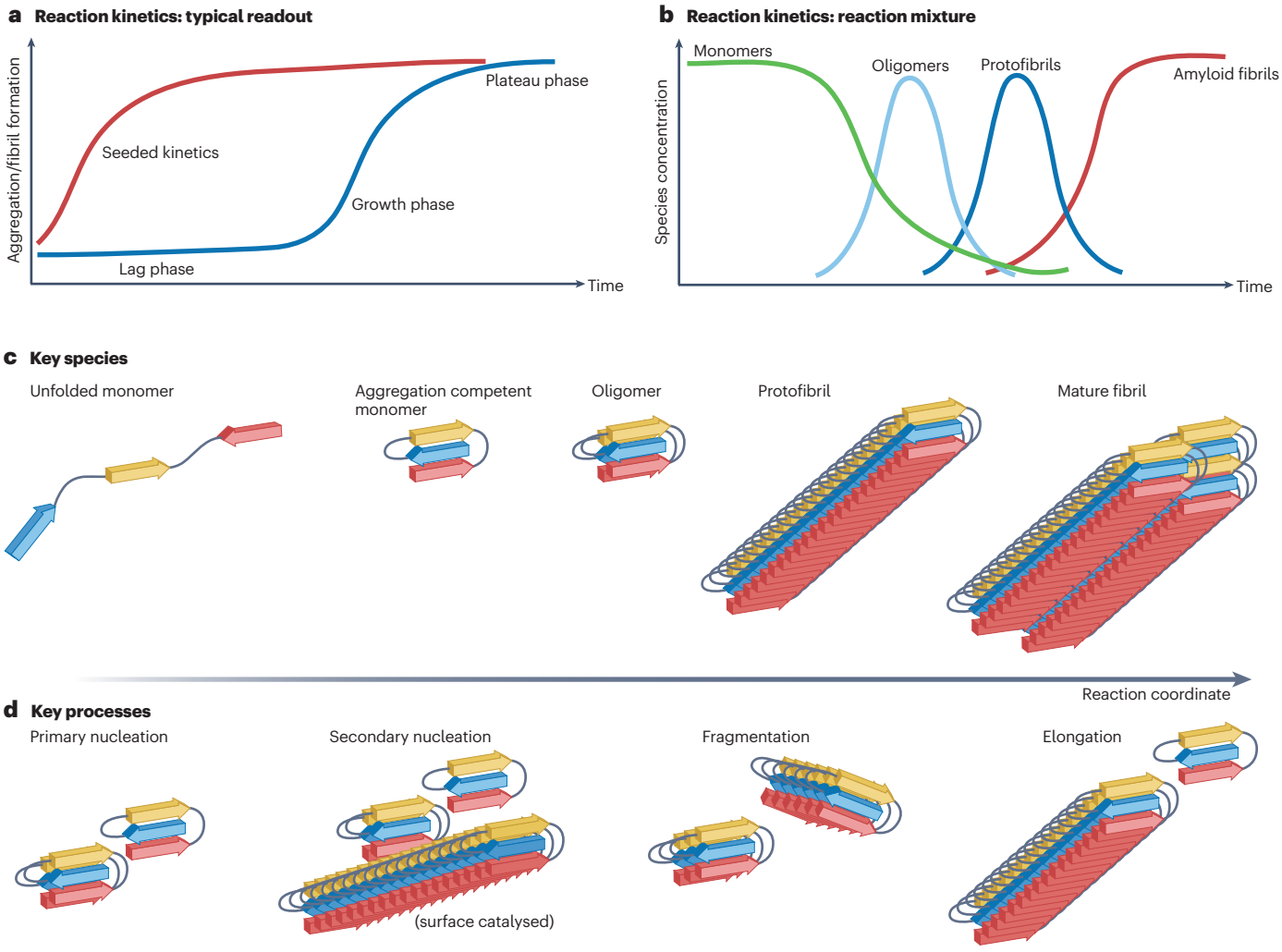
Fig 4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Amyloid fibrils
Time
c Key species
Unfolded monomer
Aggregation competent monomer
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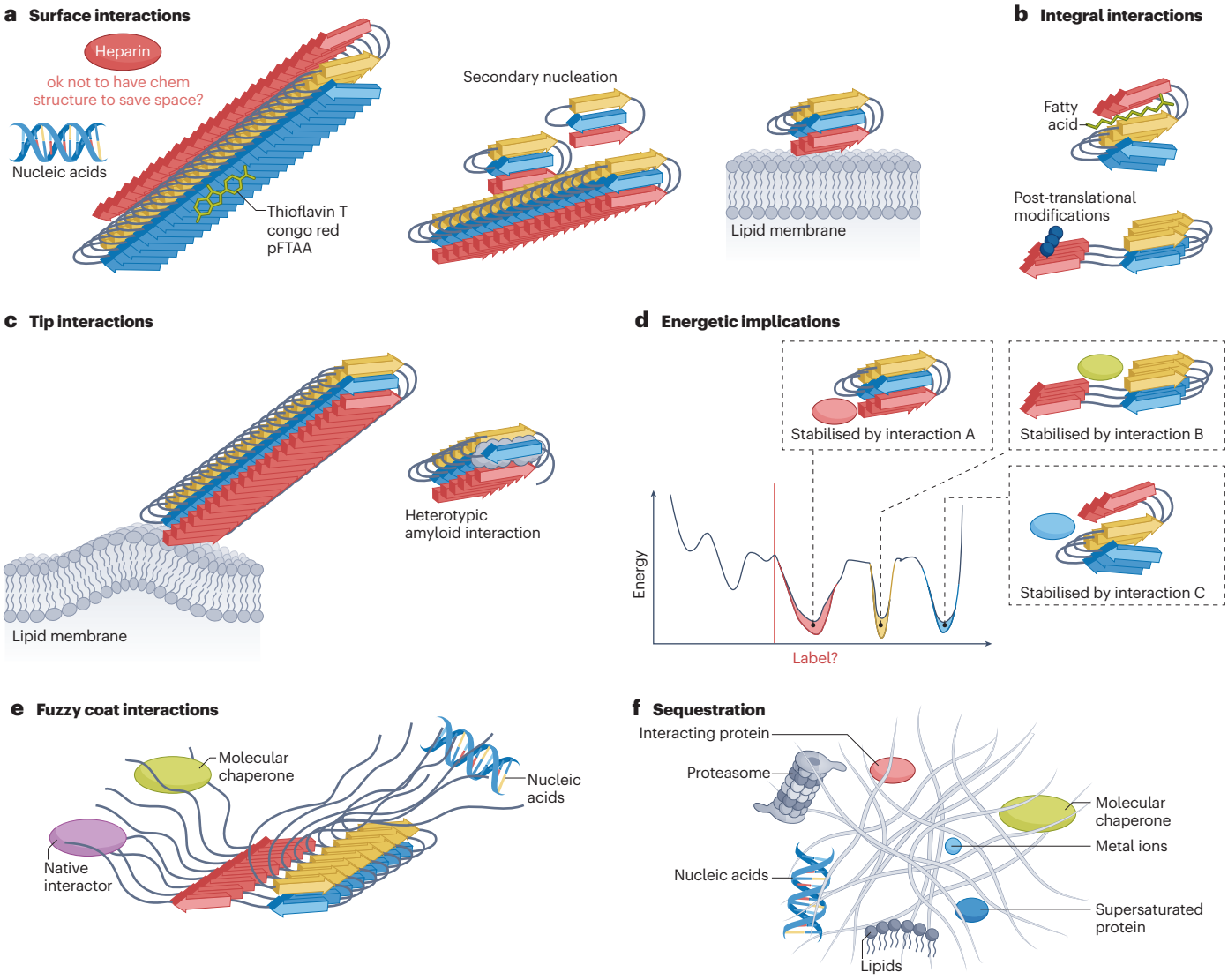
Fig 5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