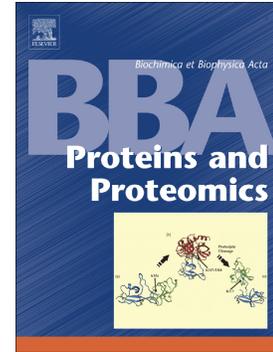


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**The molecular lifecycle of amyloid – mechanism of assembly,
mesoscopic organisation, polymorphism, suprastructures, and
biological consequences**

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ABSTRACT

The formation of a diverse range of amyloid structures from normally soluble proteins and peptides is a hallmark of devastating human disorders as well as biological functions. The current molecular understanding of the amyloid lifecycle reveals four processes central to their growth and propagation: primary nucleation, elongation, secondary nucleation and division. However, these processes result in a wide range of cross- β packing and filament arrangements, including diverse assemblies formed from identical monomeric precursors with the same amino acid sequences. Here, we review current structural and mechanistic understanding of amyloid self-assembly, and discuss how mesoscopic, i.e. micrometre to nanometre, organisation of amyloid give rise to suprastructural features that may be the key link between the polymorphic amyloid structures and the biological response they elicit. A greater understanding of the mechanisms governing suprastructure formation will guide future strategies to combat amyloid associated disorders and to use and control the amyloid quaternary structure in synthetic biology and materials applications.

Keywords: amyloid; prions; polymorphism; suprastructure; self-assembly

Introduction

Amyloid forming proteins are at the centre of various protein misfolding disorders as well as having normal physiological functions in a wide variety of organisms from bacteria to mammals. The main characteristic of amyloid forming proteins is their propensity to form ordered fibrils with a core made of tightly packed β -sheets perpendicular to the fibril axis. The organisation of soluble peptides or proteins into insoluble amyloid fibrils has been identified in many human pathologies [1], including amyloid β ($A\beta$) and tau involved in Alzheimer's disease (AD), α -synuclein (α -syn) in Parkinson's disease, and huntingtin in Huntington's disease. Other amyloid-associated disorders include type II diabetes and several types of systemic amyloidoses [2–4]. However, not all amyloid structures are disease associated. In fact, a range of non-disease associated amyloid structures participate in an array of normal physiological processes without any apparent harmful effects to their hosts [5,6]. Thus, it is difficult to establish direct causal relationships between amyloid protein precursors, the large range of structures they form, and the diseases they are associated with due to a lack of evidence linking structural and mechanistic understanding of neurodegenerative disease aetiology.

The building blocks of amyloid fibrils are soluble monomeric proteins or peptides. Their primary sequences typically include amyloidogenic motifs containing amino acids with high propensity to assembly into amyloid fibrils, influenced by both the physiochemical properties of these amino acids, as well as their order in the sequence. These motifs tend to be hydrophobic, more rigid and have a tendency to form β secondary structures [7]. Despite differences in the primary sequence between amyloid-forming proteins, the monomeric

precursors form fibrils with a characteristic cross- β structure in all amyloid [8,9]. The cross- β architecture that defines the amyloid core is composed of β -strands packed perpendicularly to the fibril axis, and can be readily observed by X-ray fibre diffraction usually showing a characteristic 4.7 Å reflection on the meridian of the diffraction pattern that corresponds to the spacing between β -strands and indicates that they are stacked perpendicular to the fibril axis. The β -sheets can be arranged in parallel or antiparallel arrangements, forming a stable cross- β configuration that resists degradation by proteases, detergents and heat. Pairs of β -sheets usually intermesh with close side chain complementarity in a variety of possible steric zipper arrangements [10]. The ~ 10 Å equatorial reflection corresponds to the spacing between β -sheets and indicates that there are usually two or more sheets in amyloid filaments [9]. The supramolecular fibril structure is supported by intermolecular hydrogen bonds parallel to the fibril axis, making amyloid fibrils strong fibrous materials [11]. Amyloid fibrils typically have diameters of approximately 5-20 nm and can be up to several micrometres long [12–14].

The structure of amyloid fibrils has been studied to high detail using atomic force microscopy (AFM), solid state NMR spectroscopy (ssNMR) and cryo-electron microscopy (cryo-EM), which provide information on β -strand content within a monomer, arrangements of β -sheets, specific interactions between residues and the conformation of non- β -strand segments. In particular, the advent of high resolution cryo-EM and ssNMR methods has led to the elucidation of a range of amyloid species at near-atomic resolution, including several formed from the same monomeric precursors (**Fig. 1**). In this case, differences in fibril forming conditions, such as pH, temperature and salt concentration affect the morphology of fibrils formed. In fact, identical monomers under the same conditions can often form a mix of highly polymorphic suprastructures, and the heterogeneity exhibited by amyloid samples

complicates structural and functional studies while their mechanistic origins and biological consequences are not understood.

The aggregation pathway by which soluble monomers form oligomeric intermediates and eventually fibrils is thought to be similar for all amyloid, despite differences in primary sequences and pathological presentation. The aggregation process starts with protein misfolding events in which native state monomers adopt amyloidogenic states and aggregate into nuclei that grow into oligomeric intermediates of increasing size distributions. Monomers are then added to the oligomeric intermediates, forming larger, more flexible, often elongated oligomers, frequently called protofibrils. These intermediate species are then lengthened into ordered assemblies of cross- β filaments that can elongate further with the addition of monomers at their ends. At the same time, two or more filaments can associate to form fibrils. Amyloid fibrils can further cluster into a variety of suprastructures, which include extracellular plaques, as in the case of $A\beta$, or intracellular inclusions, as in the case of huntingtin and tau.

Despite the shared core cross- β architecture, the detailed structures and surface features of oligomeric intermediates, fibrils and suprastructures depend on the specific precursor protein, and they elicit varied biological effects [15]. For example, polymorphs of *in vitro* formed $A\beta_{40}$ can have different levels of toxicity on neuronal cell cultures [16]. Amyloid fibrils are then able to undergo division, for example by fragmentation through mechanical stress, catalysis by specific cellular components such as chaperones [17], or due to biochemical changes in the cellular environment, into shorter fibril particles that act as seeds. The seeds are further elongated by monomers, which are continuously produced by their host organism. Thus, rather than a linear process, amyloid assembly represents a molecular lifecycle in

which a ‘cloud’ of species and suprastructures in a heterogeneous mixture are continuously being produced, and the species populations in this cycle will evolve as a function of time in response to changes in the conditions.

This review will discuss the molecular lifecycle of amyloid assembly in terms of the current understandings of the key molecular processes involved. It remains poorly understood how some amyloid aggregates are tolerated or even beneficial while others, despite having similar core structures are associated with debilitating neurodegenerative diseases. Moreover, identical monomer sequences can also form fibrils with different morphological and phenotypic consequences, adding complexity to finding the structural determinants behind amyloid aggregation, toxicity, and biological response. The wide degree of heterogeneity and structural polymorphism of amyloid fibrils will be discussed in this review to demonstrate that all species in the amyloid lifecycle are an integral part of the lifecycle and form a population that may contribute to the pathogenic potential of amyloid as a whole. Finally, this review will also address in what manner the suprastructural arrangement of amyloid assemblies may represent a fundamental link between amyloid structures and their functional variations in biological systems. This will be discussed in terms of how suprastructures may affect fibril division and propagation of the amyloid state in the amyloid lifecycle, as well as how they affect the infectious and cytotoxic potentials of amyloid.

The amyloid lifecycle and its defining molecular processes

The molecular details of amyloid fibril formation are debated, but the self-assembly reaction fundamentally consists of four key processes: nucleation, elongation, division [18] and

secondary nucleation [19] (**Fig. 2**). However, the current description of amyloid aggregation and the resulting fibril assembly pathway is influenced by *in vitro* kinetic studies where the reactions are limited by the amount of monomers present. This process is better represented as a lifecycle, considering that *in vivo*, monomer production, misfolding, nucleation, elongation, division and secondary nucleation all occur continuously and simultaneously, and it is their relative rates that change over time as defined by the microscopic rate constants associated with each step. It should also be noted that all of the steps along the amyloid lifecycle are dynamic and reversible, although some reverse reactions are associated with high kinetic barriers.

In the initial stages of the amyloid lifecycle, partial unfolding and/or conformational changes are required to convert amyloidogenic proteins in their native states to misfolded states capable of further conversion into the amyloid state. The monomeric or small oligomeric amyloid precursors initially exist in a dynamic equilibrium of conformations with varying degrees of structural order and can aggregate to form small amyloid oligomers. These intermediate oligomers can be structurally similar to fibrils in their conformation and β -sheet content as shown by binding of conformation-specific antibodies and analysis of secondary structure content. They can also be highly disordered, or indeed anywhere in-between [20]. Thus, at some point along these initial aggregation events, monomers or small oligomers adopt a conformation with a high β -content. This process can be accelerated by specific mutations and environmental factors. This initial aggregation process of primary nucleation can be seen as a phase transition of the amyloidogenic protein from an aqueous solution phase to the 'solid phase' represented by amyloid fibrils. Primary nucleation can proceed as homogenous nucleation occurring in solution, or heterogeneous nucleation occurring on surfaces or interfaces. As biological environments are rich in surfaces, it is possible that

many spontaneous primary amyloid nucleation events occur as heterogeneous nucleation *in vivo*. Thus, surfaces may have profound effects on aggregation kinetics depending on their composition and properties [21]. Nucleation events generally occur after a “critical concentration” of monomers in solution has been reached and exceeded [22,23]; these solutions are called supersaturated solutions [24]. The smallest oligomeric aggregate on which further growth is more likely to occur than reduction in size is called the nucleus, although the generic nature of amyloid nuclei remains unresolved.

Once formed, amyloid nuclei can grow by templated elongation, in which free monomers are converted to the amyloid state and added to growing filament ends. Although elongation is a reversible reaction, dissociation of monomers is usually negligible due to the highly stable fibril structure contributing to a slower relative detachment rate compared to the attachment rate during elongation. Nucleation and elongation are concentration-dependent processes [25], and their relative contributions to amyloid formation varies between different amyloid proteins and solution conditions [23,26,27]. Post-nucleation species capable of templating elongation growth are called seeds. Seeds can grow into protofilaments, which are elongated structures with monomeric units in the amyloid state. Protofilaments can subsequently twist around each other forming fibrils. Thus, fibril ends act as the growth active sites of amyloid fibrils. A fibril typically may consist of 2-6 protofilaments, and can be further elongated and adopt a more ordered fibril arrangement. Some fibrils are quite flexible and can circularise and grow into loops [28]. Adding to the ambiguity of amyloid formation terminology, there is no objective definition for “mature” fibrils. This term is typically used to refer to long and straight fibrils observed in an end-stage *in vitro* assembly reaction, but there is no formal length, width or twist definition for these.

In the *in vivo* amyloid lifecycle, the large and possibly biologically inert fibrillary aggregates will be part of a heterogeneous population of aggregates of a range of sizes and states, including small intermediate oligomeric species, often referred to simply as ‘oligomers’. There is currently no universal definition of what constitutes an amyloid oligomer, but common features include a ‘soluble’ (i.e. not true soluble in a physicochemical sense but small enough to be dispersed and not sedimented by centrifugation), heterogeneous and transient nature. Such small oligomeric amyloid aggregates vary in subunit composition and morphology, for example, disordered, spherical and annular structures having been identified *in vitro* [29–31]. Major oligomer types include fibrillar oligomers, which structurally and immunochemically closely resemble short fibril particles, and prefibrillar oligomers, which are intermediate species having a distinct but not well characterised structure [32]. There is also a type of oligomer that is formed off-pathway from fibril formation that may be highly cytotoxic *in vitro* [33].

Additional complexity arises from the fact that oligomer populations are inherently highly polymorphic. Oligomeric structures have been determined under different conditions using diverse techniques, and it is not always clear whether, or in what form, they exist *in vivo*. This raises the question of what the toxic physicochemical or structural properties of amyloid species might be. In addition to the formation of amyloid species, which represents a phase transition of protein precursors in aqueous solution to an insoluble solid phase, amorphous aggregates with no ordered cross- β core may instead result from transition to a liquid phase, forming liquid droplets through liquid-liquid phase separation (LLPS). It has been hypothesized that the LLPS process is utilised by cells to compartmentalise proteins and biochemical reactions and consequently has physiological roles in cell signalling and regulation of gene expression [34]. Such liquid phase separated structures include P granules, nucleoli and stress granules, and are typically made up of aggregates of nucleic acids and

protein. Intrinsically disordered proteins are also often found in cellular liquid droplets as their exposed hydrophobic areas and structural freedom may facilitate aggregation. Similarly, amyloid liquid-liquid demixing could be promoted by molecular chaperones [35]. Demixing allows high local concentrations of specific proteins, and as the droplets stabilise and mature over time, they may provide a driving force to further phase transition to a solid phase, characterised by amyloid fibril formation.

Lateral sides of protofilaments or fibrils are able to catalyse the formation of new amyloid nuclei and oligomeric species capable of growth by monomer addition in a process known as secondary nucleation [27,36]. Secondary nucleation is a special case of heterogeneous nucleation where the catalysing surface is specifically that of preformed amyloid instead of any surface. New nuclei formed through this secondary process then detach and can be further elongated. Indeed, it has been suggested that once a critical concentration of fibrils has been reached, fibril-catalysed secondary nucleation becomes the major source of toxic oligomeric species [27]. Despite secondary nucleation events occurring on pre-existing fibrils, amyloid fibrils are not considered to grow into branched suprastructures as each protofilament in a fibril remains unbranched.

Finally, amyloid fibrils are capable of dividing into smaller fibril particles. The division of amyloid fibrils propagates the amyloid state and the conformation associated with the parent fibril assemblies [18]. Amyloid division can occur due to fibril fragmentation caused by thermal energy or mechanical forces, or be catalysed enzymatically by chaperone proteins [37]. Division increases the number of fibrils particles and, therefore, reactive fibril ends that can lead to further growth by elongation. For prions, which are amyloid that are transmissible between hosts, division of amyloid fibrils is required for infectivity and propagation of the

prion phenotype [38], which suggests a similar mechanism could occur in prion-like amyloids correlated with neurodegenerative disease. As a single prion or amyloid forming sequence can assemble into a wide range of fibril polymorphs, the differences in their ability to divide may result in the selection of specific prion strains under specific corresponding conditions. These properties will affect phenotype strength of the prions and will depend on the structural stability of the amyloid fibrils. Thus, fibril stability changes may alter its propensity for division which, in turn, affects amyloid toxicity and prion infectivity [39]. The biological implications of these structural differences could reveal a key element of the amyloid structure-function relationship.

Mapping the kinetics mechanisms of amyloid assembly

The complex nature of amyloid aggregation kinetics due to non-linear growth processes in the amyloid lifecycle, combined with their high sensitivity to environmental and experimental factors, has challenged the development of kinetic assays and derivation of rate laws. While the outline of the amyloid lifecycle as discussed above is generally well understood and documented based on *in vitro* assembly experiments, the specific structures of the species involved and the rates of their formation and exchange remain unclear.

Amyloid assembly *in vitro*

Currently, the kinetics of amyloid self-assembly is frequently assayed *in vitro* utilising the tinctorial property of amyloid following development of high-throughput microplate-based kinetic assays [25] using the dye thioflavin T (ThT). ThT shows enhanced fluorescence emission upon binding to in-register side-chains within the β -sheets of amyloid fibrils, and a

kinetic assay with ThT as amyloid reporter is widely used as it is simple and relatively sensitive compared to turbidity and light scattering approaches [40]. ThT assays have allowed in-depth studies of amyloid formation kinetics and derivation of rate laws that have elucidated the molecular mechanisms of amyloid assembly and aid in determining the mode of action of fibril formation inhibitors [41]. Also, ThT fluorescence intensity may reflect fibril morphology, regardless of the β -sheet content due to different accessibility to binding sites [42].

Although ThT is a useful tool for quantifying the relative amounts of fibrillar cross- β content over time, it does have some limitations and shortcomings. For example, ThT cannot be used for specific identification of amyloid fibrils, as it is not sufficiently specific to amyloid aggregates and can for example, bind to DNA [43], nor can it be used to distinguish between amyloid fibrils and prefibrillar species [44]. Additionally, screening the effect of small molecule inhibitors on fibril formation using ThT is prone to false positives as the candidate inhibitors may interfere with the binding of the dye, rather than the fibrillation process [45]. The study of amyloid aggregation should always be complemented with various other biophysical techniques, including circular dichroism (CD) and Fourier transfer infrared spectroscopy (FTIR) for secondary structure characterisation, and AFM and EM for fibril imaging. Several derivatives of ThT have been developed for *in vivo* detection of amyloid fibrils in the organs and tissues of live patients [46,47]. Amyloid probes also include luminescent conjugated oligothiophenes, which are fluorescent amyloid ligands that can report on the fibrillar conformation, facilitating the *in vitro* and *ex vivo* analysis of polymorphism [48,49]. The continued development of novel reporters for use in animal models and in future clinical applications will contribute to a better understanding of the formation and spread of amyloid aggregates under *in vivo* conditions.

The main distinctive characteristic of amyloid aggregation, as measured *in vitro* using ThT, which gives a fluorescent signal increase upon binding amyloid fibrils, is a sigmoidal growth curve (**Fig. 3**). The lag phase represents early reaction times where primary nucleation events that lead to nuclei and small intermediate oligomer formation dominates. Nucleation is initially thermodynamically unfavourable and kinetically rate limiting as the nucleation process is associated with a free-energy barrier, with pre-nucleation species in the reaction coordinate favouring dissociation compared to further growth by monomer association [25,50,51]. The lag phase can be eliminated by introducing preformed fibrillar amyloid seeds, enabling the conversion and addition of monomers directly to fibril ends in the amyloid state, thereby bypassing the rate-limiting nucleation process. At the end of the lag phase, while most of the protein is still monomeric, there is a transient population of intermediate oligomeric species, some of which are referred to as protofibrils because they are sufficiently and observably elongated species [52]. Small transient oligomers grow and fully convert into protofilaments with amyloid cross- β core that are then elongated by further addition of monomers to fibril ends. As fibrils form during lag phase, secondary processes such as secondary nucleation and fibril fragmentation become the major mechanism of amyloid formation, peaking during the exponential growth phase [25,27]. Fibril mass increases as protofilaments are elongated and twist around each other. Finally, growth plateaus as most monomers in solution have been added to fibril ends. The population in such a sample remains in dynamic exchange (**Fig. 2**). For example, monomers and oligomers may continuously break off and reattach to fibril ends [53]. Importantly, these key steps are part of the lifecycle of all amyloid fibril formation, but their rate constants and thermodynamic driving forces vary for different monomers [50].

Amyloid assembly *in vivo*

While the fundamental kinetic principles governing the self-assembly of amyloid are the same *in vitro* as *in vivo*, the kinetics of the amyloid lifecycle under *in vivo* conditions would be expected to be significantly different from the well-characterised *in vitro* conditions. One of the main differences is the unlimited and continuous production of monomers *in vivo*. Other key differences include the added complexities associated with genetic variations of amyloid monomer or its precursor and other risk factors, components of the cellular and extracellular environment that affect amyloid aggregation kinetics, spatiotemporal variations in monomer production affecting local monomer concentrations, and amyloid clearance mechanisms. Using fluorescence lifetime imaging (FLIM), the kinetics of A β aggregation, as well as its cellular uptake and trafficking, have been studied in live neuronal cells [54]. The aggregation of A β in human APP-expressing mice was analysed using a fluorescent amyloid dye and a cranial window through which images were collected using two-photon imaging over a period of two years [55]. The plaque volume change was found to have a sigmoidal shape, with many small plaques forming initially, when the concentration of free A β is high, and growing in volume until they plateau when A β production becomes rate-limiting. However, while transgenic murine models of AD are useful research tools for familial AD, they fail to represent the most abundant type of AD in humans, which is sporadic. Amyloid positron emission tomography (PET), a method for visualizing amyloid deposition in the brain using radiopharmaceuticals that bind fibrillar amyloid, has also been used to collect and assemble A β load data from cognitively impaired patients to create a long-term disease model that showed a sigmoidal curve of increasing amyloid load in the brain over the course of more than 30 years [56]. Overall, few studies have been done on the *in vivo* aggregation kinetics of amyloid in animal models or humans in any molecular detail.

The rates governing the amyloid lifecycle and its kinetics are specific to protein sequence and environmental conditions. For example, aggregation propensity is affected by charge and exposed hydrophobic surfaces of the monomer. The lag phase can also be shortened by an increased fragmentation propensity. Although a key feature of many amyloid-forming proteins is being intrinsically disordered as monomers, or having significant stretch of unstructured regions due to extensive exposed hydrophobic areas, others can be folded, globular, and with low aggregation-propensity in their native states [57]. Such proteins, including β_2 microglobulin associated with systemic dialysis related amyloidosis, require local unfolding to initiate aggregation [58]. In these cases, physiological thermal fluctuations may be sufficient for native conformation destabilisation [59] and hence enough for initiating assembly. Conditions such as temperature, pH, protein-denaturing agents, presence of other proteins, metal ions, surfaces and their composition and properties can have an impact on the aggregation of amyloid proteins [21,60–63]. Even many physiologically non-amyloidogenic proteins can be made to adopt the characteristic cross- β structure under specific environmental conditions [64,65]. Deviations from the typical kinetic characteristics of amyloid aggregation have been identified under varying environmental conditions, which has implications for the biomedical use of amyloid-prone proteins, and for developing drugs targeting amyloid assembly. For example, the human glucagon-like peptide 1, analogues of which are used for treatment of type II diabetes, exhibits kinetics consistent with the standard nucleation-polymerisation mechanism at pH 8.2, but at pH 7.5 the kinetics showed a highly unusual profile, with the lag phase becoming longer with increasing monomer concentration [66]. This was attributed to the formation of off-pathway oligomers, with unknown physiological effects. One plausible explanation is that metastable oligomers and protofibrils could be off-pathway competitors and inhibitors of fibril formation, instead of on-pathway precursors [67].

Amyloid assembly polymorphism

Amyloid fibrils, by definition, share a cross- β core arrangement. Their assembly, either from precursors of different or identical sequences, nevertheless result in fibrils with a varying degree of structural differences (**Fig. 1, Fig. 4**). Polymorphism of molecular crystals has been characterised in the context of small molecules and pharmaceuticals, for which the varying physiochemical properties including the stability and bioavailability of a substance with an identical molecular structure and dissimilar suprastructure has been documented [68,69]. Analogously, the fibrillar amyloid state can be viewed as a pseudo one dimensional “crystal-like” form in which different polymorphs may also have varying biological properties. Variance in fibril structure, i.e. the polymorphic nature of amyloid fibrils, has been characterised for fibrils formed from synthetic or recombinant monomeric amyloid precursors, as well as those formed *in vivo* in tissue or by seeding with fibrils from brain tissue of patients with various neurodegenerative diseases [70–72]. Amyloid assembly polymorphism resulting from assembly of precursors of identical sequence can be broadly divided into two classes: core polymorphism with differences in the arrangement of monomeric units in the cross- β core, or filament polymorphism with differences in the lateral arrangement of protofilaments in a fibril and the specific contacts they form (**Fig. 4**). In core polymorphism, the core structure can vary in β -sheet content, conformation of non- β -strand segments, steric zipper packing and specific contacts between residues. Core polymorphism can be further categorised into segment polymorphism where different segments of a polypeptide may form different cross- β cores, and packing polymorphism where the same segment of a polypeptide chain is involved in the cross- β core. For example, inter-sheet

contacts can be stabilised by steric zippers, as well as hydrophobic contacts and salt bridges. Eight potential classes of steric zipper packing arrangements were described by Eisenberg and colleagues [10] forming the basis for core and assembly polymorphisms. These steric zipper arrangements vary by whether β -strands that make up β -sheets are parallel or antiparallel, whether adjacent β -sheets that form the steric zipper pack same or different surfaces together, or whether the β -sheets themselves are oriented parallel or antiparallel relative to each other. In terms of filament polymorphism, the current confirmed examples include cryo-EM reconstructions of purified paired helical and straight tau filaments, which show indistinguishable cross- β and β -helical structures, surrounded by a fuzzy coat of disordered domains, but distinctive inter-protofilament arrangements [73] and β_2 -microglobulin, for which several morphologies were identified using cryo-EM, although all shared the same core structure, as shown by NMR [74].

Amyloid populations regularly contain heterogeneous mixtures of fibril polymorphs. Often, several subpopulations of amyloid fibril polymorphs can form under identical conditions, for example paired helical and straight fibrils of tau or striated ribbon and twisted fibrils of A β [73,75]. In studies in which a single fibril structure is reconstructed, the sample may have contained a broad range of morphologies, as in the case of the recent β_2 -microglobulin structure [74]. These individual fibril polymorphs can sometimes be distinguished by their width, as they may vary in the number of protofilaments (**Fig. 4**), or other morphological differences such as twist periodicity and persistence length in terms of curvature. Additionally, fibril polymorphs may vary in stability and dynamic behaviour which may, for example, affect their fragmentation rate and consequently cytotoxicity [76]. Fibrils differing in twist and length can also result in differences in their cytotoxic potential [16,39].

From fibril structures determined from patient brain tissue, it emerges that there may be disease-specific association with certain amyloid fibril polymorphs. For instance, tau fibrils from an Alzheimer's patient and a Pick's disease patient have remarkably different core arrangements [72,73]. Additionally, structural polymorphs of A β are thought to correlate with variations in AD pathological phenotypes [77]. For example, recent ssNMR analysis of A β fibrils seeded from AD patient brain tissue showed a link between clinical AD subtypes and specific features of fibril polymorphism. A β_{40} aggregates were shown to have a single major morphology in patients with typical prolonged-duration AD and posterior cortical atrophy variant (PCA-AD) and a higher proportion of alternative structures in the rapidly progressive form of AD. By contrast, A β_{42} aggregates were found as several polymorphs across both categories [77]. In another study, A β_{40} fibrils seeded from brain tissue of two AD patients with different clinical histories were relatively homogenous for the individual patients, although analysis of the predominant fibril structures by ssNMR between the two patients indicated significant differences in their cores [78].

Due to amyloid and prions have structural and mechanistic similarities, and the morphological heterogeneity of amyloid populations potentially linked with disease progression, the idea of whether structural amyloid polymorphs propagate phenotypically as strains has been tested. For prions, the prion strains give rise to specific pathologies and disease phenotypes, which are maintained when the strains are introduced *de novo* into a genetically identical host where they continue to be stably propagated. Some studies have also suggested the spread of tau and α -synuclein as distinct strains [79]. Thus, identifying the structure of distinct fibril polymorphs, the suprastructures they form, and characterising the environmental factors that drive structural changes, as well as their biological effects, could be the key link to elucidating the dramatic variations in amyloid disease pathological

presentation and provide a structural rationalisation of the strain phenomenon. The structural differences between amyloid polymorphs could also be mediated by changing the propensity to the various fibril associated pathology mechanisms, including interactions with membranes, ability to sequester proteins essential for the cell, differences in metal binding and creation of reactive oxygen species, or some as yet unknown mechanism of fibril toxicity. Thus, in terms of disease association, environmental changes may modulate amyloid structure and exert pressure to select for specific polymorphs, and the selected polymorphs in turn reinforce the disease-associated environmental changes.

Amyloid suprastructures

As amyloid fibrils are highly polymorphic, the heterogeneous populations of amyloid species that result from the amyloid lifecycle also show a variety of different mesoscopic arrangements on the micrometre to nanometre scale, forming a variety of possible suprastructures (**Fig. 5**). Amyloid fibrils vary in width, with some self-associating into thick bundles by protofilament interactions, whereas other fibrils may consist of a single protofilament only [80]. The length distribution of the fibrils in a population can similarly vary, depending on the mechanical properties of the fibrils, such as stiffness, and their fragmentation rate [18]. Each fibril population formed from the same amyloid sequence may contain varying proportions of fibril polymorphs, ranging from a uniform ensemble to almost continuous variation in twist [13,78]. While some fibrils self-associate into packed clusters or networks, others do not form such structures and remain separated, sometimes in a parallel alignment with directional order [81,82]. Although various suprastructures have been identified, little is known about their relative biological impact. At liquid interfaces amyloid

fibrils can exist in a liquid crystalline nematic phase in which the fibrils are aligned parallel to each other [83]. *In vitro* studies of entangled amyloid networks have also identified gel-like behaviour at the mesoscopic scale. Interestingly, elastic properties appear as early as during nucleation events in the lag phase [84]. Electron tomography studies of A β suprastructures have revealed three main types of aggregates: amorphous meshwork, fibril bundle and amyloid star, all within the same overall deposit [85]. Interestingly in each case, the fibrils themselves were morphologically indistinguishable. Additionally amyloid fibrils have been observed to form spherulites which show a typical Maltese cross pattern when observed under polarised light in both *in vitro* and *ex vivo* [86,87]. Furthermore, between fibril networks, extracellular vesicles of various sizes are found [85] and lipid membrane components have been identified also within and around dense A β plaques in human brains of AD patients [88].

Morphological differences observed in plaque deposits reflect the suprastructural assembly preferences and features of amyloid aggregates, and seeded fibrils from AD patient brains have been correlated to differences in clinical subtypes [77]. Thus, it is possible that the different suprastructures that differ in their mesoscopic arrangements also have different toxic or infectivity profiles. The suprastructural arrangements of fibrils can also be affected by the dominance of individual fibril polymorphs as they can have different surface properties which affect their interactions. Thus, aggregates with different suprastructural features will also have different surface properties and propensities to sequester other metastable essential constituents of the cellular proteome. Characterising the various suprastructural parameters such as length and width distribution, twist, stiffness, clustering and heterogeneity of amyloid assemblies and correlating these with biological activity could lead to essential insights into the amyloid structure-function relationship.

Regulation of functional and pathological amyloid structure and assembly

Defining an amyloid suprastructure-toxicity relationship would help us elucidate why some amyloid formations are highly toxic whereas others are inert. Given the presence of amyloid aggregates in numerous neurodegenerative diseases, it is perhaps surprising that an increasing number of amyloid structures have been found to participate in an array of normal physiological processes without any observable harmful effects to their hosts [6]. In humans these include melanin biosynthesis, regulation of long-term potentiation (LTP) and peptide hormone storage [89–91]. Functional amyloid assemblies have also been discovered in numerous other organisms, including bacteria, fungi and metazoa. For instance, in some bacteria, fibrillar matrix of extracellular amyloid proteins such as curli and Fap are required for surface adhesion and colony formation [92–95]. In insects and fish, the eggshell is primarily made up of chorion proteins with a characteristic amyloid fibril structure [6]. The line between functional and pathogenic amyloids is also increasingly blurred, as subtle changes in processing or regulation may cause an amyloid with normal physiological roles to become pathogenic. For example, A β is produced from the amyloid precursor protein (APP) in neural and other cells throughout the human lifetime [96]. The precursor can be cleaved by α - and γ -secretases leading to production of non-amyloidogenic fragments or by β - and γ -secretases, which produces several isoforms of A β correlated with AD. However, there is evidence that monomeric A β and other peptides resulting from precursor cleavage might have important roles in cellular signalling pathways, regulating synaptic activity and might even be essential for survival of neurons [97]. Moreover, fibrillar forms of A β have been suggested to have protective effects against fungal and bacterial infections in mouse,

nematode and cell culture models of Alzheimer's disease, thus suggesting that they might have a role in innate immunity [98]. Recently, human neural cell culture models have also been used to investigate the role of a herpes simplex 1 virus infection on amyloid aggregation and it was reported that A β oligomers bind virus surface glycoproteins and mediate resistance to the virus [99]. This finding suggests that even small oligomeric amyloid species may have functional roles.

Amyloidogenic proteins lack sequence homology and can have diverse structural and catalytic functions in their normal non-amyloid states. The ability to self-assemble into an amyloid state could be a generic structural feature of polypeptide chains [100]. Therefore, understanding the mechanistic and structural differences between functional and pathogenically-associated amyloid, which share the same cross- β core structure by definition, is a key requirement for treating amyloid-associated diseases as any potential treatment must be able to recognise essential functional features of amyloid and differentiate these from the pathogenically-associated features. A key aspect of functional amyloid may lie in their controlled and localised assembly initiation and termination in response to environmental cues, which is sometimes achieved with post-translational modifications. For example, the CPEB3 protein activates the transcription of mRNAs that promote long-term potentiation (LTP), but only when ubiquitylated and deSUMOylated, which promotes its assembly into the functional fibrillar form [90,101]. SUMOylation of CPEB3 makes it soluble and inactive, although in other amyloid proteins it can promote aggregation and toxicity [102]. Additionally, many peptide hormones that are stored within endocrine granules require both the compartmentalised acidic pH environment and the presence of glycosaminoglycans (GAGs) for fibril formation. GAGs accelerate fibril formation by abolishing the lag-phase and some can interfere with potentially harmful fibril-membrane interactions [103,104].

Another regulatory mechanism of controlling amyloid assembly involves chaperones. Recently chaperones DNAJB6 and Hsp70 were identified as part of a natural control mechanism to prevent the aggregation of α -synuclein, found as toxic aggregates in Parkinson's Disease patients [105]. α -syn is expressed at high levels in healthy individuals in various tissues of the body and, in the monomeric state, has important synaptic functions related to neurotransmitter release and synaptic plasticity, although the exact mechanisms are not known [106]. Chaperone proteins such as Hsp70, Hsp40, and others are also involved degradation and refolding of amyloid aggregates. The chaperone Hsp104 is essential for propagation of $[PSI^+]$ prion phenotype in yeast that is associated with the functional amyloid state of Sup35; Hsp104 promotes division and propagation of Sup35 amyloid by catalysing fibril fragmentation *in vivo* [107,108].

On the basis that amyloid associated with pathology may have roles in normal physiological processes, it has been suggested that it is a dysfunctional protein degradation machinery that leads to disruption in proteostasis and build-up of toxic amyloid aggregates [109]. Some functional amyloid systems, for example pre-melanosomal protein, aggregate into fibrils much more rapidly than non-functional amyloid, thus preventing accumulation of intermediate oligomeric species with potential for harmful effects [110].

All the above-mentioned mechanisms may have evolved to ensure the normal roles of functional and/or to prevent the pathogenic features of amyloid assemblies, which have important implications in numerous physiological processes involving amyloid or amyloid precursors with no deleterious effects. Safety mechanisms that control and regulate amyloid localisation, compartmentalisation, processing and degradation, as well as their assembly

kinetics and interactions with other cellular structures, may present some of the differences between functional and disease associated amyloid.

Structural and molecular origins of amyloid toxicity

Although the hallmark of neurodegenerative disease is deposits of amyloid fibrils, the neuropathological and clinical symptoms vary significantly. Therefore, the identity of the toxic species, and the molecular origin of the cytotoxic potential associated with amyloid is widely debated. For example, AD is characterised by progressive loss of synapses, neuronal death and atrophy of the affected areas, resulting in decline of memory and cognitive functions, whereas the main pathophysiological characteristic of Parkinson's disease is degeneration of dopaminergic neurons in the substantia nigra resulting in loss of motor function leading to rigidity and tremors. Other neurodegenerative and prion diseases also involve progressive neuronal death in various areas of the brain and result in different symptoms. Severe cases of amyloid deposition has also been found in the brains of human subjects with no cognitive decline or symptoms of dementia upon post-mortem assessment of neuropathology [111]. Thus, several underlying toxicity mechanisms involving a range of amyloid species have been suggested for these symptoms, including disruption of cell membranes, dysregulation of calcium homeostasis, mitochondrial dysfunction and oxidative stress.

Toxicity potential of small oligomeric amyloid species

Amyloid toxicity is currently thought to result from pathological effects associated with small amyloid oligomers as they are ubiquitous in the brains of neurodegenerative amyloid disease

patients and their cytotoxicity has been well characterised *in vitro*. According to this hypothesis, large deposits such as amyloid plaques and inclusion bodies are thought to be relatively inert. Indeed, small oligomeric species from post-mortem human brains can disrupt long-term potentiation, synaptic plasticity and memory when injected into a mouse hippocampus [112]. This view is supported by evidence of neurodegeneration and cognitive defects preceding plaque formation *in vivo* [113,114]. In the case of mutant huntingtin, the formation of inclusion bodies was also found to reduce the risk of cell death from toxic mutant huntingtin aggregates [115]. Furthermore, the formation of prefibrillar oligomers is accelerated by mutations in α -syn causing a familial, early-onset form of Parkinson's [116]. However, the precise molecular nature of the small oligomeric species that confers toxicity remains to be established and a large number of species of varied structures and suprastructures have been observed. For example, according to the 'ion channel hypothesis', oligomeric species with a ring-like structure insert into cell membranes and act as aberrant ion channels, disrupting the homeostasis of ions and leading to cell death [117]. Such oligomers have been suggested to form membrane-associated annular structures *in vitro* through interactions with specific lipids in the membrane, and possibly form a β -barrel pore [118–120]. However, specific structural features may be required for ion channel formation, as it has been suggested that oligomers of $A\beta_{42}$ but not $A\beta_{40}$ are capable of channel formation in membranes [121]. Disruption of calcium homeostasis has been observed in proximity of amyloid plaques, which may act as a reservoir of cytotoxic species [122]. Loss of calcium compartmentalisation leads to distortion of neuritic morphology and as calcium is essential for neural integration of signals, dysregulation of its homeostasis disrupts local neural networks [122]. Amyloid oligomer cytotoxicity has also been linked to the size and conformation of the oligomers, with smaller and more exposed hydrophobic surfaces with structural flexibility displaying the most toxic potential [123,124]. Thus, the dynamic and

hydrophobic nature of prefibrillar oligomers may provide a rationale to their propensity to aggregate and display cytotoxic properties through their interaction with membrane bilayers.

The elucidation of the role of small oligomeric species in amyloid diseases is complicated not only due to their varied structures and heterogeneity, but also due to their transient nature. Thus, in terms of species formed during the amyloid lifecycle *in vivo*, due to experimental limitations it is only possible to study stable species secreted by *in vitro* cell cultures or those extracted from post-mortem patients' brains. Furthermore, the population and the concentration of amyloid species in the brain is unknown. Attempts to quantify the concentration of A β in the brain has focused mostly on mouse models, with some studies on human brain tissue and cerebrospinal fluid [125–127]. However, results are inconclusive as there is currently no method for quantifying whole amyloid populations ranging from prefibrillar oligomeric species to fibrillar species *in vivo* without exposing them to non-native conditions that could affect their aggregation states, leading to unreliable estimates. Additionally, very little is known about how the local environment in the *in vivo* human brain affects amyloid structure and toxicity, especially as the amyloid population are likely to be highly heterogeneous and distinct oligomeric species can vary in toxicity.

Toxicity potential of fibrillar amyloid species

In addition to the cytotoxic effects displayed by prefibrillar oligomeric species, amyloid fibrils also have direct cytotoxic properties via disruptive effects on the phospholipid bilayer during fibril growth [128]. Localised neuronal damage, characterised by progressive neuronal dystrophy and microglial activation, has been correlated with plaque formation and shown to worsen over the clinical course of the disease [129,130]. Several indirect mechanisms of fibril toxicity have been proposed, including secondary nucleation events where fibrils

catalyse the formation of small oligomeric species through surface interactions, thereby contributing to the neurotoxic effects of amyloid [27]. Additionally, fibrils may act as a reservoir for toxic species, which may be released to generate a local pool of toxic species as a halo around fibrillar deposits [131]. Besides these indirect mechanisms, fibrils can also contribute directly to cell damage under conditions in which non-fibrillar aggregates are not detectable by spectroscopy or antibody-binding, thus suggesting a role as a direct contributor of cytotoxicity.

Fibril fragmentation is key to amyloid cytotoxicity by increasing the number of termini through division of fibrils, which provides increased reactive growth competent surface, and also creates smaller fibrillar amyloid species that decrease cell viability and increase disruptive effects on membrane bilayers. The increase in the toxic potential cannot be solely attributed to the increased number of fibril ends, suggesting other yet unknown surface-dependent mechanisms [39]. Furthermore, short fibril particles are also readily internalised by endocytosis, causing disruption within the cell by inhibiting the degradation of proteins within lysosomes and altering trafficking of lysosomal membrane proteins [132]. Disruptive interactions of fibrils with membranes have been visualised in 3D using electron tomography, surrounded by lipid inclusions of varying sizes [133]. Extraction and clustering of lipids around amyloid aggregates and their potential links to toxicity have also been previously characterised [134]. Fibril-membrane interactions also promote the shedding of oligomers from fibril ends that then diffuse rapidly through the brain and impair cognitive function in mice [135]. These oligomers formed through reverse assembly reactions were found to have a highly heterogeneous size distribution, but similar biochemical and physical properties to those formed by nucleation and forward assembly reactions [135]. Another potentially physiologically harmful effect of amyloid could arise from fibril interactions with metal ions

[136], as several co-localise with amyloid plaques in AD patient brains [137]. A β_{42} fibril interactions with copper (Cu²⁺) enable retention of redox activity and generation of reactive oxygen species *in vitro* [138]. The presence of metal ions also affects fibril aggregation morphology, which may have additional consequences on biological and pathogenic properties [136,138,139].

Modulating factors of amyloid cytotoxicity

In the complex, crowded environment of cells and nervous tissue, the cellular milieu plays an important role in affecting how the amyloid lifecycle progresses, and how amyloid species and populations form and interact with other cellular structures. Surfaces, such as those presented by membranes, can promote protein misfolding and aggregation, thus potentially speeding up nucleation events in the amyloid lifecycle, promoting the *de novo* formation of amyloid species [140]. This effect depends on the lipid composition of the membrane. Differing membrane composition could potentially explain the variable vulnerability of various cell types to toxic amyloid species. Phosphatidylserine and other acidic phospholipids could provide a local low-pH environment that promotes fibril formation [141]. Interestingly, functional amyloid assemblies are often compartmentalised into membrane-bound organelles without causing damage. A specific membrane composition could explain why this key toxicity target is not harmed by functional amyloid aggregates. Furthermore, hydrophobic surfaces and the air-water interface are also capable of inducing heterogeneous nucleation and formation of small amyloid oligomers, as well as leading to the formation of fibrils with a distinct morphology compared to fibrils formed by homologous nucleation away from surfaces [142,143].

The lack of clear correlation between amyloid fibril aggregates with clinical symptoms in neurodegenerative diseases promoted the view that small oligomeric pre-fibrillar species are the main toxic species. While some oligomeric species display significant cytotoxic potential, not all oligomeric species share this potential. In the same manner, some amyloid fibril structures possess cytotoxic potential while others appear to be inert. Difficulties in studying the mechanisms and structures associated with amyloid toxicity under physiologically relevant conditions also add to the fact that the composition of the amyloid populations that are associated with disease as a whole, and their combined modes of action, have not been resolved. It is possible that the incoherence between the amyloid species and the neurodegenerative disease symptoms and progression they are associated with could be better explained not by individual amyloid structures but the sum of their presence in a population as a whole. The variation in the composition of the heterogeneous amyloid population resulting from the amyloid lifecycle may drive different characteristics and clinical symptoms associated with amyloid.

The infectious potential of amyloid: prions and prion-like amyloid

Prions are infectious amyloid particles with the self-propagating amyloid cross- β state. Mammalian prions are correlated with a number of currently untreatable neurodegenerative diseases termed transmissible spongiform encephalopathies (TSEs) which include kuru, Creutzfeldt-Jakob disease, bovine spongiform encephalopathy, and scrapie in sheep. These diseases can arise spontaneously, be inherited, or acquired through an infection by prion particles. TSEs are caused by an amyloidogenic form of the mammalian prion protein (PrP) and can be transmitted between individuals and sometimes even across species [144,145].

However, not all prion replication is disease-associated and in fungi in particular they can have neutral or beneficial effects. For example in yeast, prions confer phenotypic plasticity through evolutionary selective advantages [146]. For example, the *Saccharomyces cerevisiae* protein Sup35 is a subunit of the eukaryotic translation release factor, required for termination of mRNA translation. The amyloid form of Sup35 is associated with the $[PSI^+]$ prion phenotype [147]. Similarly, $[URE3]$ is the prion phenotype associated with the *S. cerevisiae* Ure2 protein [147]. Both of these prion proteins can form several strain variants with different characteristics and have been studied extensively to elucidate the molecular mechanisms and structural determinants of amyloid proteins [148].

Prions have been considered a subclass of amyloid that can be transmitted between cells and organisms [149]. However, there is now increasing evidence to suggest that some pathogenic amyloid proteins can also be transmissible from cell-to-cell. For example, cross-cell transmission has been demonstrated for A β [150], α -syn [151], huntingtin [152] and tau [153], blurring the distinction between ‘prions’ and ‘amyloid’. As the likelihood of transmissibility of these pathogenic amyloid proteins between individuals, an essential aspects of prion behaviour, is probably low, they are consequently typically classified as ‘prion-like’ amyloid. Nevertheless, these proteins may be infectious to an extent, as shown for tau where injection of tau-containing brain extract of human origin can induce tau inclusions in transgenic mice expressing wild type human tau [154]. Recently, further evidence was found to support iatrogenic transmission of A β by identifying A β_{40} and A β_{42} , along with tau, in archived vials of human cadaveric pituitary-derived growth hormone [155], which was used to treat patients until 1985 when some were diagnosed with Creutzfeldt-Jacob-Disease (CJD) [156]. This raises concerns over accidental transmission of prion and

prion-like amyloid during medical procedures and through potentially amyloid-contaminated surgical equipment.

How some amyloid can show a type of prion-like behaviour is not known, but fibril fragmentation seems to play a key role in facilitating infectivity [157], as in the case of prion particles, division and propagation through fibril fragmentation could also be a key determinant for infectivity and amyloid phenotype strength [38]. Fibril fragmentation could facilitate vesicular cell-to-cell transport, due to the smaller size of fragmented particles, as cell-to-cell spreading is thought to be mediated by intracellular amyloid particles weakening the lysosomal protein degradation pathway, leading to exocytosis of oligomeric species capable of propagating the amyloid state that are then taken up by recipient cells [158]. Thus, the stability of amyloid particles towards fibril fragmentation, which may be modulated by their suprastructure, could represent a link between amyloid structure and their infective potential as prions.

Challenges in establishing amyloid structure-function relationships

Many important aspects of amyloid toxicity, propagation, and their role in neurodegenerative diseases remain elusive. A significant gap in knowledge relates to the specific mechanisms of amyloid pathogenicity in terms of the structural properties associated with the toxic and infective potential displayed by amyloid structures. To effectively develop therapeutics that specifically target toxic or infective properties or species, it is first necessary to understand the mechanisms of toxicity, transmission and propagation in detail. However, for amyloid-associated diseases it is not yet clear whether amyloid aggregates are a cause or a

consequence of the disease, and by which mechanisms they could exert toxic effects to the cells. Consequently, no safe and effective anti-amyloid treatments have yet been developed despite efforts by academia and pharmaceutical companies alike. Although aggregates of amyloid fibrils are an important hallmark for diagnosis of amyloid disease, their role in pathology is debated. It is thought that earlier species in the amyloid lifecycle, specifically the small intermediate, oligomeric, pre-fibrillary species, are responsible for some of the cytotoxic and neurodegenerative effects in amyloid associated pathologies [159]. However, there is also evidence of fibrils with a shorter length distribution having cytotoxic effects [160]. Additionally, they could have important roles in amyloid propagation and other indirect mechanisms of toxicity, as well as being involved in the infective activities associated with prions and prion-like amyloid. The problem is exacerbated by the lack of high-resolution structural information of intermediate species on or off the fibril formation pathway as they have remained largely elusive due to conformational heterogeneity and transient nature.

Often, populations containing a heterogeneous mixture of amyloid polymorphs or amyloid species from different precursor sequences can be present in the brain of an affected individual, which might be part of the complex and varied nature of neurodegenerative diseases [71,161]. As discussed above, a plethora of amyloid fibril polymorphs may form and grow under the same conditions, but each individual polymorph may have different effects on cells. However, it is often necessary to achieve a homogenous sample of fibrils of a single morphology by progressive seeding or stabilising fibrils of a specific polymorph for structural characterisation and when assaying biological effects of amyloid fibrils. A nearly homogenous population can be achieved by repeated seeding because fibrils with different morphologies have different rates of self-assembly [75]. However, the species distribution as

a whole may have an impact on the pathological properties associated with amyloid. For example, in a population, some species may contribute directly to the accelerated propagation of the amyloid state in the amyloid lifecycle and only indirectly in the accumulation of toxic species, while other species may act as cytotoxic entities directly. Whole population effects could also rest in the varied concentrations of different species in the population as the toxic potential of the whole amyloid population will be a sum of the toxic potential displayed by individual species in the population, weighted by their concentrations. Just like for any toxic substances, the classic principle “*sola dosis facit venenum*” (the dose makes the poison) will also apply to amyloid species. Furthermore, the infectious and toxic potential of amyloid will be modulated by their suprastructural states, such as clustering and filament lateral assembly, at a mesoscopic scale. This information can be obtained using - for example - atomic force spectroscopy (AFM) and transmission electron microscopy (TEM) and will be highly complementary to higher resolution structural information on individual filament types obtainable by ssNMR and cryo-EM.

Various anti-amyloid therapeutics targeting different processes in the amyloid lifecycle have reached clinical trials although so far none have been successful. Part of the problem lies with the fact that the holistic role of amyloid lifecycle in disease mechanisms has not been fully elucidated. Potential mechanisms to target amyloid formation include kinetic stabilisation of native and inert states, inhibitors of enzymes that process amyloid precursors, sequestering small toxic oligomeric species with antibodies, inhibition of amyloid-membrane interactions, prevention of elongation through blocking fibril ends, and potentially increasing elongation rates to force smaller, more toxic, states to form part of longer fibrils with less cytotoxic potential. For example, a molecular chaperone BRICHOS can effectively bind to the surface of A β fibrils, thus preventing secondary nucleation [162,163]. Engineered and enhanced

disaggregases, including Hsp104, are capable of clearing amyloid inclusions and could also potentially lead to development of therapeutics that upregulate amyloid aggregate clearance [164]. Immunotherapies targeting A β oligomers or fibrils have been thus far been unsuccessful, although results are yet to emerge for their ability to prevent disease in asymptomatic people with a genetic predisposition [165]. This highlights the fact that developing drugs for amyloid disease treatments is not as straightforward as designing an inhibitor for monomer production. A deeper holistic and systems understanding of the effects of pathogenic amyloid in the biological context is required for effective development of therapeutics.

The potential for exploiting functional amyloid as natural bio-nanomaterials has inspired the development and rational design of artificial nanomaterials which use their unique materials properties for a variety of prospective applications in biotechnology and biomedicine. The self-assembly mechanism produces highly stable fibrils with a tensile strength comparable to that of steel [166]. These properties make them highly lucrative for novel biomaterial development. Additionally, amyloidogenic proteins are amenable to significant sequence and chemical modifications to alter their physio-chemical properties as the fibrils are able to maintain their structure under a wide range of conditions. Potential applications of synthetic amyloids include mechanisms of drug delivery as a reservoir for controlled release of drugs, and tissue repair as a molecular scaffold promoting cell adhesion, migration and differentiation. Another possible use of amyloid fibrils includes forming biosensors by entrapping proteins or other sensory molecules, depending on the desired application, or the formation of nanowires by forming long hollow tubes [167]. Amyloid-carbon hybrid membranes have also been developed for inexpensive water purification, efficiently removing heavy metal ions and radioactive waste [168]. Thus, if bespoke amyloid can be

designed and negatively selected against molecular features that are associated with toxic properties, amyloid fibrils will make excellent nanomaterials for biotechnology and biomedical purposes.

Closing remarks

Despite increasing research efforts, the specific role of amyloid structures in neurodegenerative disease remains elusive. There is still a lack of clear understanding of the identity of the toxic amyloid species, their mechanism of action and their infectious potential in relation to their structural properties. Indeed, high-resolution structural models of amyloid fibrils have now been resolved using emergent ssNMR and cryo-EM methodologies, and these advances confirm that despite sharing the same cross- β core characteristics, amyloidogenic proteins form fibrils with differences in the arrangements of the steric zipper core packing, β -sheet content, and the number and packing arrangement of protofilaments. The formation of different polymorphs and the heterogeneity of the amyloid populations can be affected by environmental conditions, and fibrils and small oligomeric species with different morphologies can form under the same conditions, including *in vivo* and in disease-affected patients. There is also now increasing evidence to show that fibrils are not inert end-stage structures, but are an integral member of the amyloid lifecycle. Through division, replication and propagation processes such as enzyme-catalysed fibril fragmentation, several potential pathways of toxicity can have key roles in the amyloid lifecycle and their pathological effects. Simultaneously, there is a large degree of variability in the biological roles of amyloid, ranging from those essential for physiological functions to those associated with debilitating neurodegenerative diseases.

Variability in fibril polymorphs and their suprastructures on the mesoscopic scale could rationalise the variations in functional and pathogenic consequences of amyloid. Thus, conflicting evidence regarding varied cytotoxicity of amyloid fibrils could be resolved by fibrils having different levels of stability, structural rigidity, surface properties, and suprastructural formations that affect cytotoxicity, aggregation, interactions with chaperones, propensity to shed oligomers or sequester essential cellular proteins. This could also rationalise why amyloid deposits in the brain do not always correlate well with clinical symptoms of neurodegenerative disease or how some amyloids can have important physiological roles without any harmful effects. Additionally, patients with the same neurodegenerative disease form different predominant types of polymorphs of the same amyloid protein that could influence disease progression and clinical symptoms. Thus, a key challenge is to establish a correlation between amyloid structure, specific mechanisms of toxicity, and variability in clinical symptoms. Fully understanding mechanisms of the amyloid lifecycle and the behaviour of heterogeneous and polymorphous amyloid populations and their suprastructural properties in the mesoscopic scale is essential to ensure the efficacy of future therapeutics targeting amyloid as well as biotechnological applications of amyloids.

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Table

Table 1: Glossary of commonly used terms relevant to the amyloid lifecycle shown in Fig 1.

Term	Description
Elongation	Growth of fibrils in a direction parallel to the fibril axis by templated monomer addition at fibril ends.
Fibril ends	Active sites where elongation by templated monomer addition occur.
Fibril fragmentation	Breakage or division of fibrils, which can be mediated by mechanical agitation, thermal stress, chemical perturbation or chaperone catalysis.
Fibrils	Long filaments formed typically by two or more intertwined proto-filaments, sometimes loosely referred to as “mature” fibrils.
Nuclei	Smallest oligomeric aggregate on which further growth by attachment of a new monomers is faster than detachment of an existing monomer in the aggregate.
Oligomers	There is no universal definition but commonly sub-100-mer aggregates featuring a heterogeneous and transient nature, and small enough to be disperse and not sedimented by centrifugation.
Primary nucleation	<i>De novo</i> formation of the amyloid state through nucleated assembly of soluble monomers either in solution (primary homogeneous nucleation), or at surfaces or interfaces (primary heterogeneous nucleation).
Proto-filaments	Single elongated amyloid filament with a cross- β core structure.
Proto-fibrils	Curve-linear or worm-like fibrils that are less ordered and elongated compared to fibrils, thought to be structurally comparable to some oligomers.
Secondary nucleation	Special case of heterogeneous nucleation in which nucleation events occur on the surface of already existing fibrils.
Secondary processes	Secondary nucleation and fibril fragmentation, which result in the

	acceleration and exponential growth of amyloid, as opposed to primary processes of primary nucleation and elongation.
Seeds	Aggregates that are capable of accelerating amyloid assembly reactions, for example post-nucleation amyloid species capable of growth by elongation, or species capable of promoting secondary nucleation.

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Figures

Figure 1: Recent structural models of amyloid fibrils determined using ssNMR and cryo-EM. These structures illustrate the similarities in cross- β core structure despite distinct primary sequences, as well as the different polymorphic structures that identical monomeric precursors can assemble into. PDB structures are shown with a yellow or orange backbone, blue basic side chain residues, red acidic side chain residues, grey hydrophobic side chain residues and green polar side chain residues. The models are oriented so that the fibrils are viewed from their ends and two layers along the fibril axis are shown for each fibril model for clarity. From upper left to lower right, the structures are drawn in Pymol from PDB entries: 6A6B [169], 6H6B [170], 6CU7 [12], 6CU8 [12], 2N0A [171], 2MXU [172], 2NAO [173], 5OQV [174], 5KK3 [175], 6GK3 [74], 5O3L [73], 5O3T [73], and 6GX5 [72].

Figure 2: Schematic illustration of the amyloid lifecycle. Soluble monomeric proteins (circles) are continuously generated and can adopt the amyloid state with a cross- β conformation (parallelograms). Coloured arrows represent the four main processes in amyloid assembly: primary nucleation (red), which may occur as homogeneous nucleation in solution or heterogeneous nucleation at interfaces; secondary nucleation (purple), which may occur as heterogeneous nucleation on fibril surfaces; elongation, which is growth at fibril ends (blue); and fibril division, for example through spontaneous or catalysed fibril fragmentation (yellow). See Table 1 for glossary of terms associated with the amyloid lifecycle. The arrows represent dynamic and reversible steps along the lifecycle.

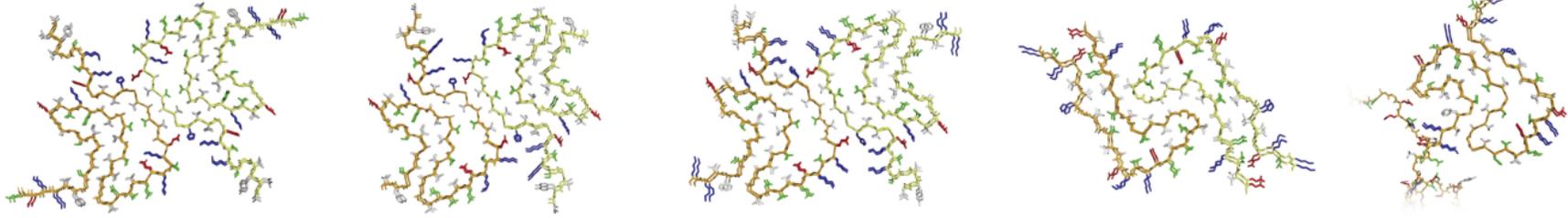
Figure 3: Schematic illustration of the amyloid aggregation growth curve as measured in vitro with kinetic ThT assay. Coloured arrows represent the four main processes in amyloid assembly (**Fig. 1**). Primary nucleation (red) is the driver of de novo amyloid aggregation in the initial stages of the reaction, although primary nucleation will continue to

take place, at much lower rates, in the later stages as free monomer concentration drop. The rate of elongation growth at fibril ends (blue) peaks during the exponential growth phase of the fibrils. Elongation continues to occur once a plateau has been reached as the fibrils are in dynamic equilibrium with residual monomers and/or small oligomers, as well as other species along the fibril formation pathway. Secondary nucleation (purple), which requires both the presence of monomers and fibrils, dominates nuclei formation as soon as first fibrils have formed in the lag phase. Fibril division through fragmentation (yellow) occurs continuously after the formation of first fibrils and continues to have a significant role in the capacity to increase fibril load. The relative magnitudes of the rates of these main processes (exemplified by the thickness of the arrows) also vary by the type of amyloid monomer, their concentration and environmental conditions.

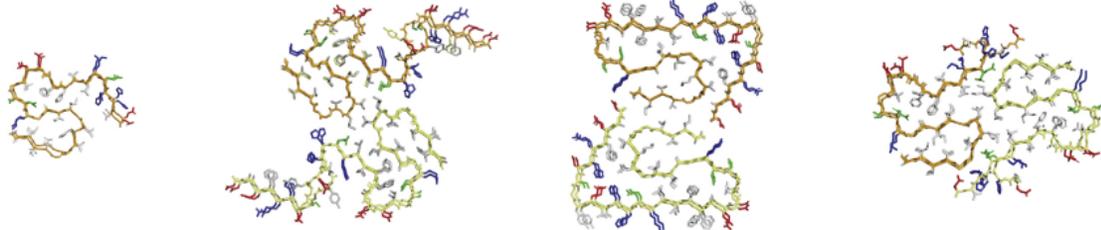
Figure 4: Hierarchical classification of amyloid polymorphism types with schematic illustrations exemplifying each type. Amyloid polymorphs can be classified into sequence polymorphs and assembly polymorphs. Assembly polymorphs can be further divided into core polymorphs and filament polymorphs, and core polymorphs can be divided in turn into segmental polymorphs and packing polymorphs. The different types of polymorphism are organised from top to bottom to indicate the hierarchical effects of polymorphism types. For example, sequence polymorphs where one polymorph contains a single amino acid residue change (top schematic, stars depict an amino acid sequence variation) would also affect which segment of the chain forms the amyloid core (segmental polymorphism), how the amyloid core is packed (packing polymorphism) and how the protofilaments are arranged (filament polymorphism). On the other hand, polymorphism based on the varied number and arrangement of the protofilaments (bottom schematic depicting filament polymorphism) may occur without changes in the sequence, the core segment or the packing of the core (all placed above filament polymorphism in the schematic).

Figure 5. Schematic illustration of possible variations in the suprastructural properties of amyloid at mesoscopic (micrometre to nanometre) length scales. a) Long-straight fibrils of varying length; b) highly heterogeneous population with filaments and bundles decorated with small oligomeric species. c) fibrils of tight twist properties d) highly fragmented fibril population with abundance of small particles; e) fibril cluster f) fibril network; g) aligned fibrils h) thick and bundled fibrils; i) flexible or curve-linear fibrils with low persistence length; j) crystalline-like fibril bundles.

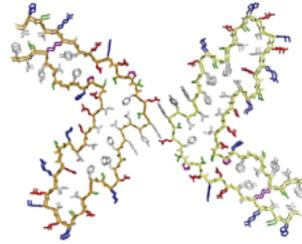
α -synuclein



A β ₁₋₄₂



β ₂-microglobulin



Tau

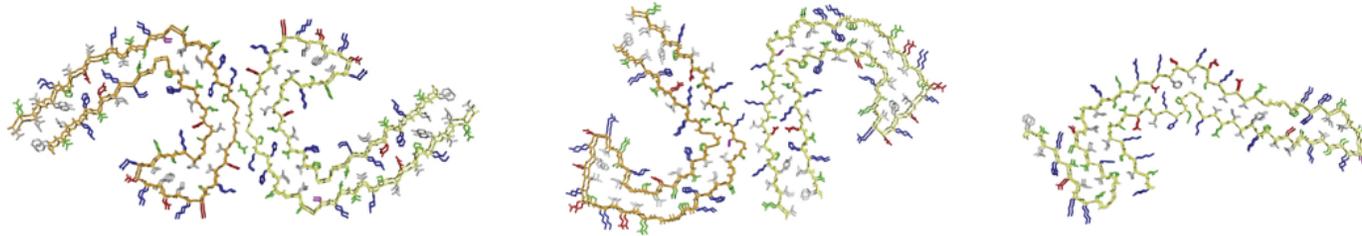


Figure 1

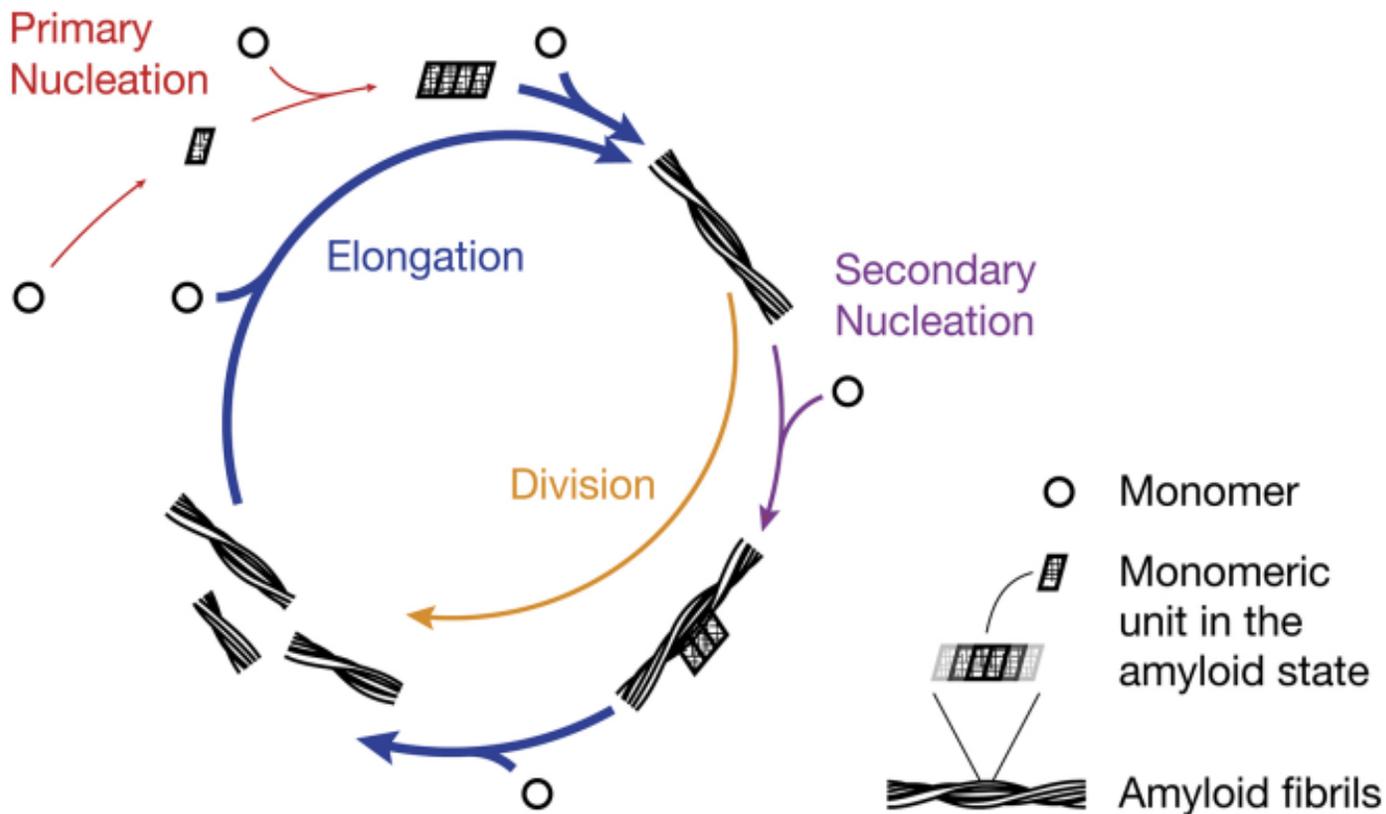


Figure 2

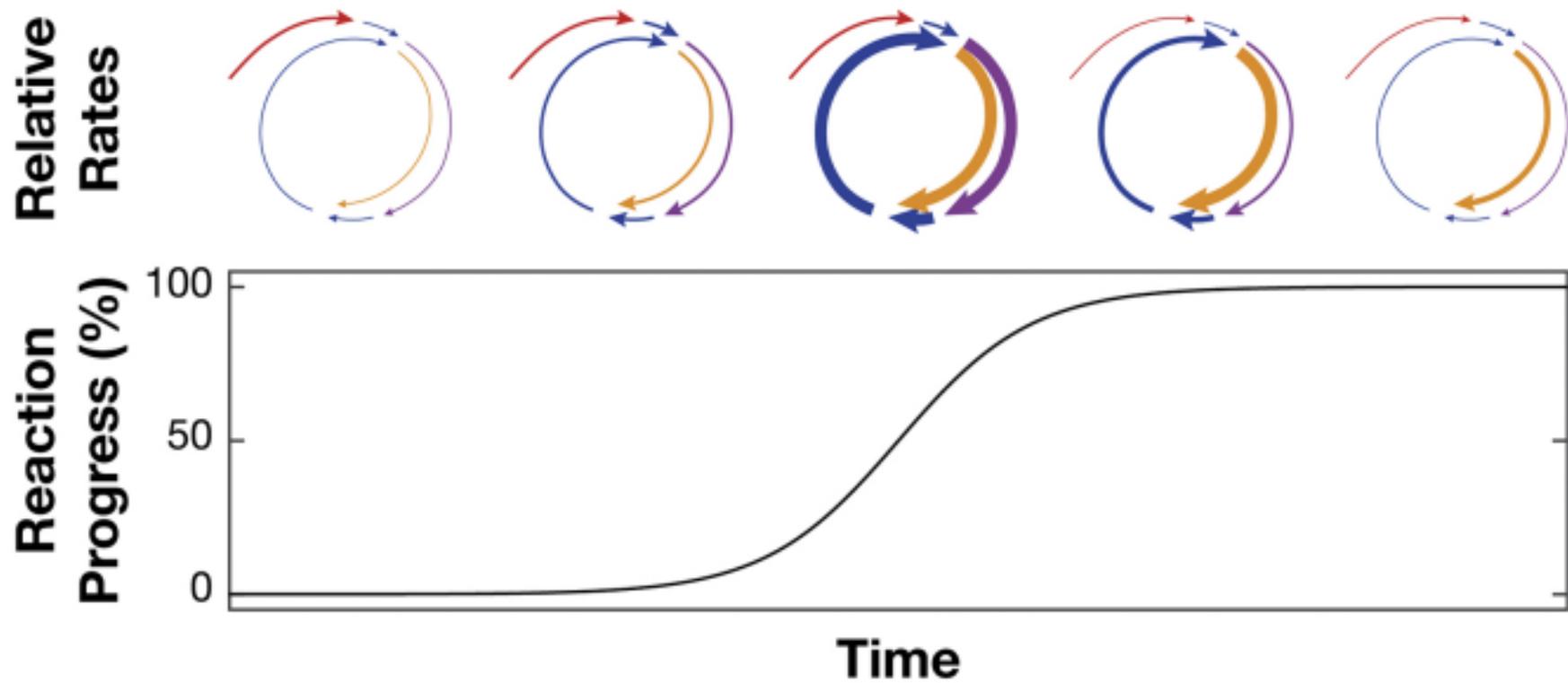


Figure 3

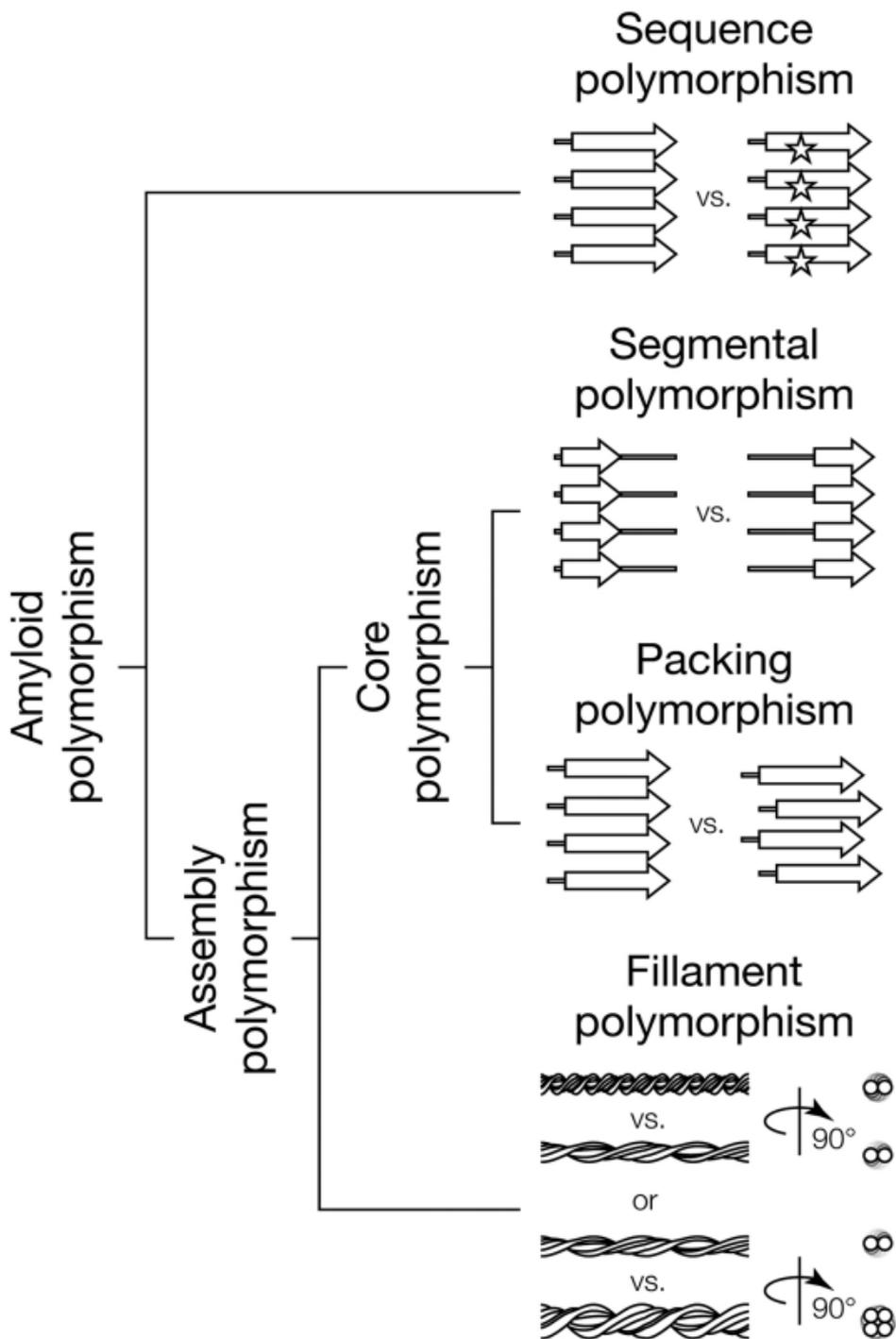


Figure 4

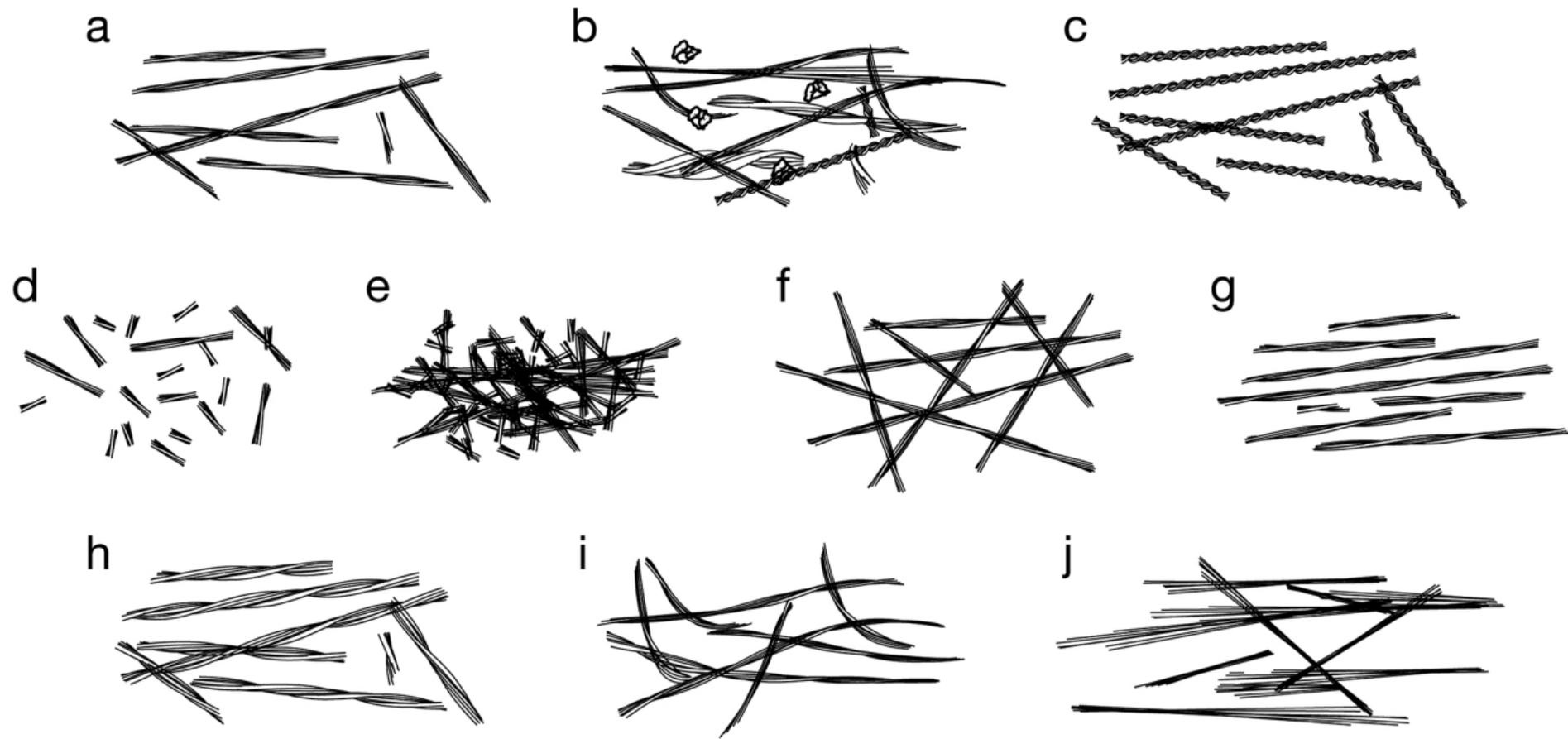


Figure 5