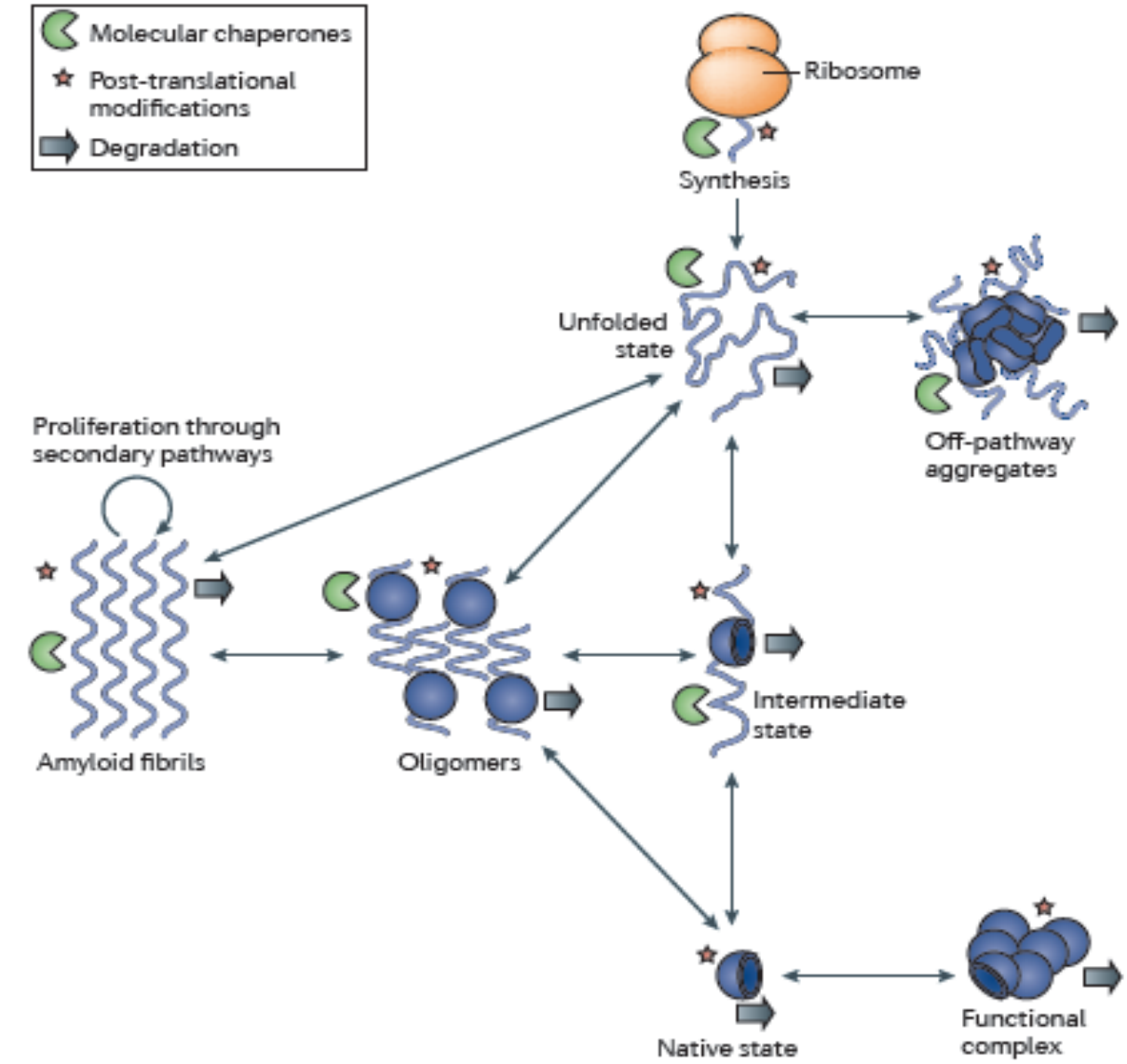
The amyloid state and its association with protein misfolding diseases

There are approximately 50 disorders, with a multitude of disparate symptoms, which are associated with the misfolding of normally soluble, functional peptides and proteins, and their subsequent conversion into intractable aggregates, of which the archetypal examples are amyloid fibrils

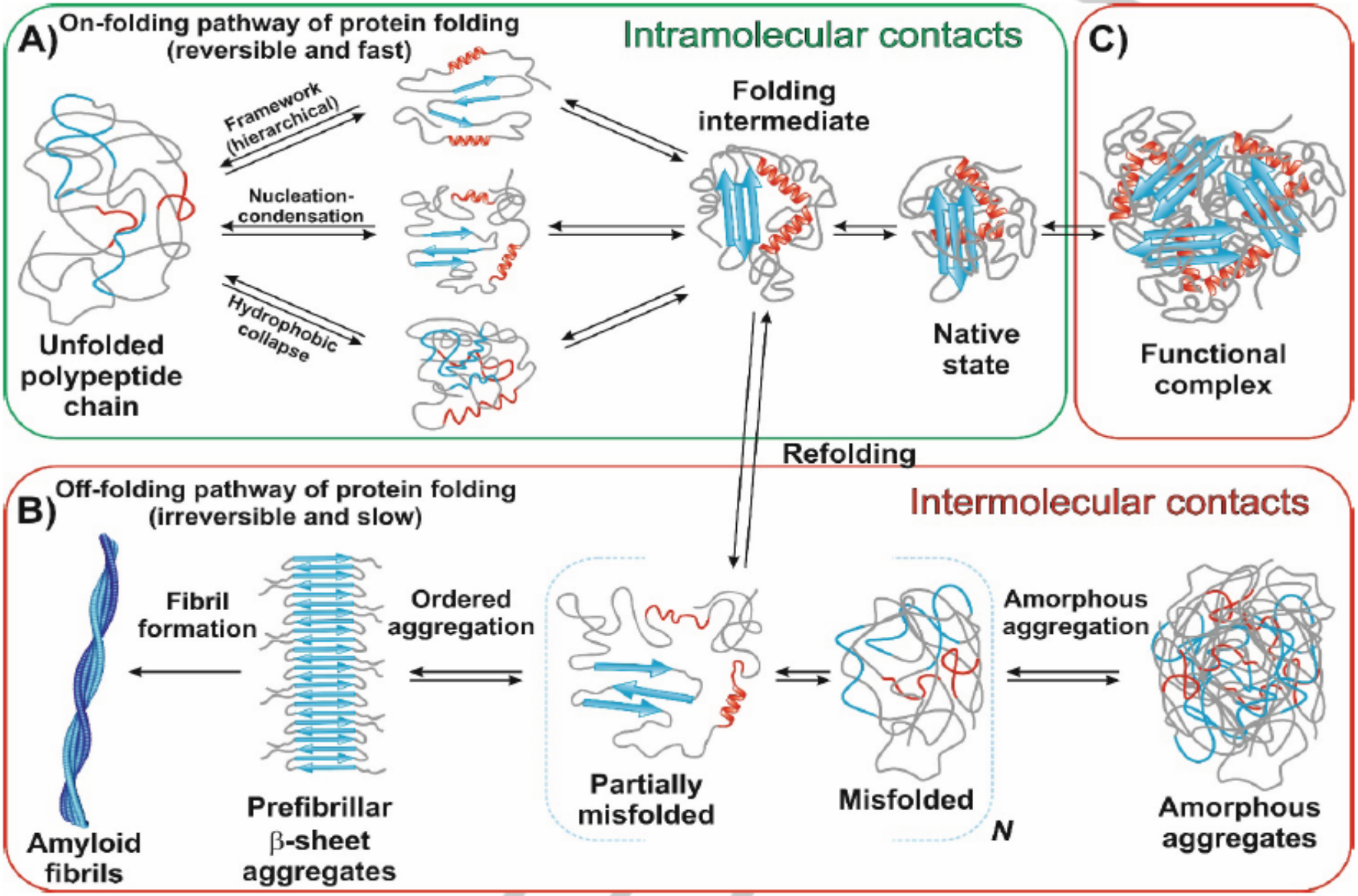
Table 1 | **Some human diseases associated with protein misfolding and amyloid aggregation***

Disease	Aggregating protein or peptide	Polypeptide length (number of residues)	Structure of protein or peptide
Neurodegenerative diseases			
Alzheimer's disease	Amyloid-β peptide	37–43	Intrinsically disordered
Spongiform encephalopathies	Prion protein or its fragments	230	Intrinsically disordered and α-helical
Parkinson's disease	α-synuclein	140	Intrinsically disordered
Amyotrophic lateral sclerosis	Superoxide dismutase 1	153	β-sheet and Ig-like
Huntington's disease	Huntingtin fragments	Variable	Mostly intrinsically disordered
Familial amyloidotic polyneuropathy	Transthyretin mutants	127	β-sheet
Non-neuropathic systemic amyloidosis			
Amyloid light chain (AL) amyloidosis	Immunoglobulin (Ig) light chains or its fragments	~90	β-sheet and Ig-like
Amyloid A (AA) amyloidosis	Serum amyloid A1 protein fragments	76–104	α-helical and unknown fold
Senile systemic amyloidosis	Wild-type transthyretin	127	β-sheet
Haemodialysis-related amyloidosis	β_2-microglobulin	99	β-sheet and Ig-like
Lysozyme amyloidosis	Lysozyme mutants	130	α-helical and β-sheet
Non-neuropathic localized amyloidosis			
Apolipoprotein A1 (Apo A-1) amyloidosis	Apo A-1 fragments	80–93	Intrinsically disordered
Type II diabetes	Amylin	37	Intrinsically disordered
Injection-localized amyloidosis	Insulin	21 and 30	α-helical and insulin-like



The amyloid state of a protein is a highly ordered form of aggregate in which the polypeptide chains adopt a fibrillar structure, which is capable of self-replication, for example through secondary processes. Amyloid fibrils are rich in β-sheet structure and typically form from unfolded or partially unfolded conformations of proteins and peptides, some of which are fragments of larger proteins.

The amyloid state is 'generic' in that its characteristic architecture is not encoded by specific amino acid sequences.



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Legend to the previous picture:

If, during the folding process, the folding intermediate of the polypeptide chain is refolding into incorrect conformations (*misfolded or partially misfolded states*), intermolecular interactions start to dominate, the misfolded chains accumulate and initiate the off-folding pathway of protein folding (aggregation process).

The aggregation process follows either the route of disordered aggregation, resulting in the formation of amorphous aggregates without defined shape, or the route of ordered aggregation resulting in the assembly of ordered fibrillar aggregates, i.e. amyloid fibrils.

The amyloid state, however, is relevant not only in the context of disease, but also because **its very existence** challenges in many ways our current understanding of the nature, structure and evolution of the functional states of proteins.

From a wide range of *in vitro* experiments on peptides and proteins we now know that the **formation of amyloid structures** is **not a rare** phenomenon associated with a small number of diseases but rather that it **reflects a well-defined structural form** of the protein that is an **alternative** to the native state — a form that may in principle be adopted by many, if not all, polypeptide sequences

As we already know, some peptides and proteins, even under normal physiological conditions, do not fold into globular structures, or they do so only in certain regions of their sequences or in the presence of specific binding partners.

Although such systems are not suitable for conventional X-ray diffraction studies, alternative biophysical methods — particularly approaches based on **NMR spectroscopy** — can provide detailed information about the ensembles of structures that exist under specific conditions.

Such proteins, often described as '**intrinsically disordered**' or '**natively unfolded**', have important biological roles, notably in signalling and regulation, not least because they are capable of interacting in different ways with multiple partners

Intrinsically disordered proteins are not, however, necessarily prone to aggregation, as their sequences have usually evolved to maintain the level of solubility that is required for their optimal function; for example, through the existence of extensive regions that are **highly abundant in charged** and **polar groups** that disfavour intermolecular association from a thermodynamic point of view. Moreover, as we know, **kinetic barriers** to aggregation are crucial in enabling both globular and disordered proteins to maintain their soluble and functional states.

Many of the peptides and proteins that are involved in the most common misfolding diseases are **intrinsically disordered** in their free soluble forms such as the **amyloid-β peptide** in Alzheimer's disease, **α-synuclein** in Parkinson's disease and **amylin (also known as IAPP**) in type II diabetes

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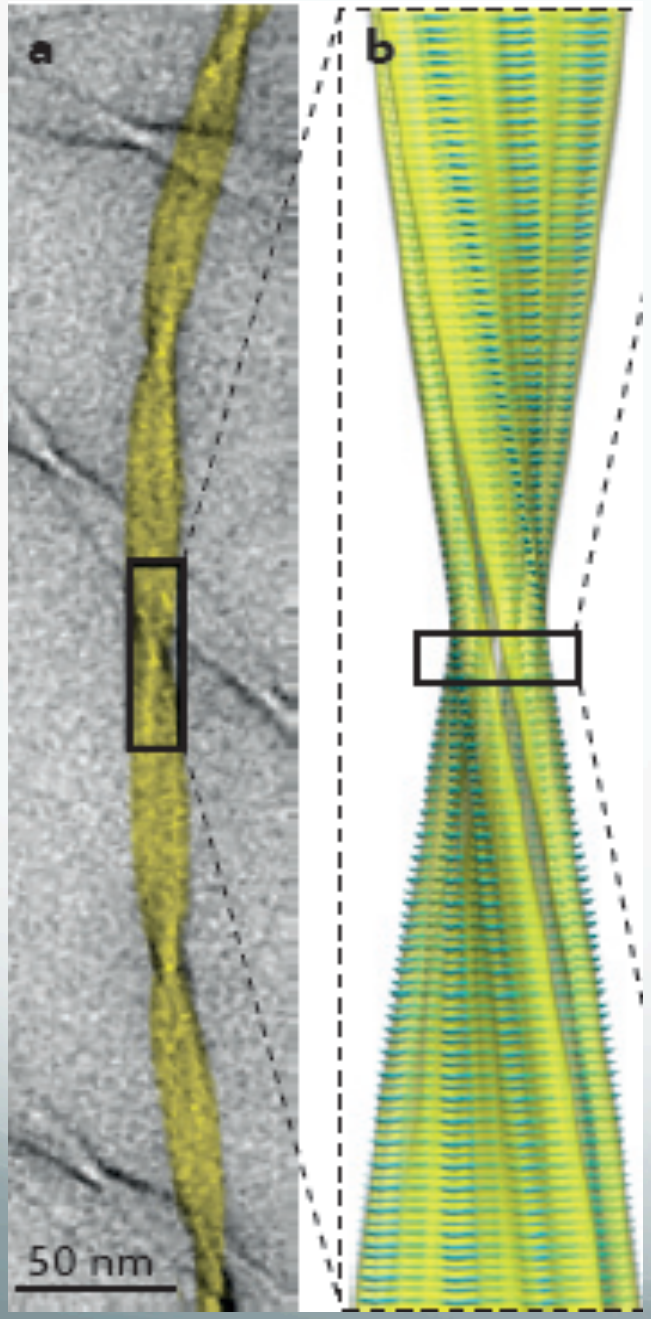
The nature of the amyloid state

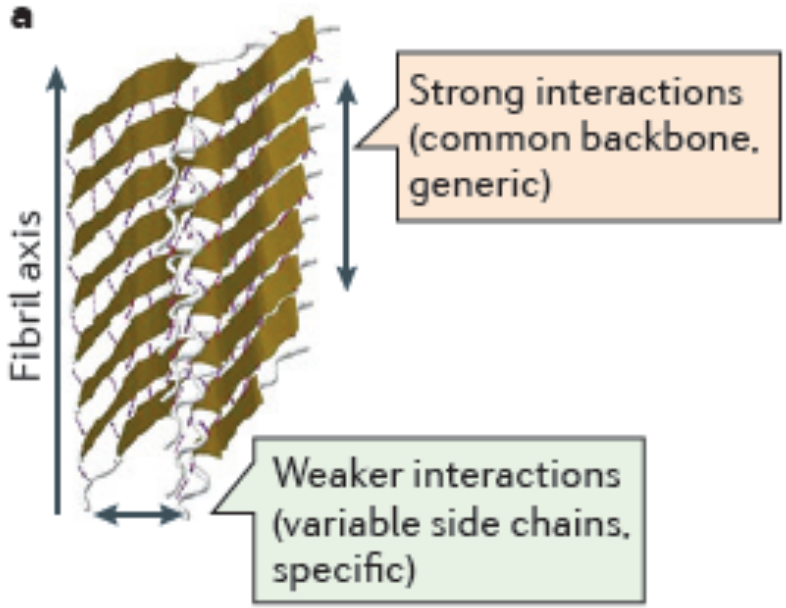
Amyloid structures have different features from native conformations, which confer unique properties upon them, typically including a very high level of kinetic and thermodynamic stability.

Detailed structure determination Similar to globular native states, amyloid structures are **closely packed** and **highly ordered**. At the same time, however, they fundamentally differ from native states, as they possess a generic architecture that is **rich in β-sheet structure**. By contrast, the folds of native states are highly diverse and can range from mostly α-helical to primarily β-sheet structures with a wide variety of different and often highly intricate topologies.



Amyloid fibrils from different proteins seem to be **remarkably similar** at the nanometre length scale. Indeed, under electron microscopy or atomic force microscopy (AFM), amyloid fibrils tend to appear as **unbranched filamentous structures** only a few nanometers in diameter but often micrometres in length.

They are typically observed to consist of multiple **protofilaments** that twist around each other to form mature fibrils



X-ray fibre diffraction studies indicate that the core of each protofilament adopts a **cross-β structure**, in which β-strands form effectively continuous hydrogen-bonded β-sheets that run along the length of the fibril.

Developments in **cryo-electron microscopy and solid-state NMR spectroscopy**, have resulted in an increasingly detailed knowledge of the molecular structures of amyloid fibrils.

These studies confirm the generic nature of the overall structures of different fibrils, which can be attributed to the **common properties of the polypeptide backbone** that support the hydrogen bonding pattern in the fibril core and to variations that result from the manner in which the different sets of side chains are incorporated into the common fibrillar architecture.

The cross-β architecture provides very **great stability** to the fibrils, as it allows the formation of a continuous array of hydrogen bonds.

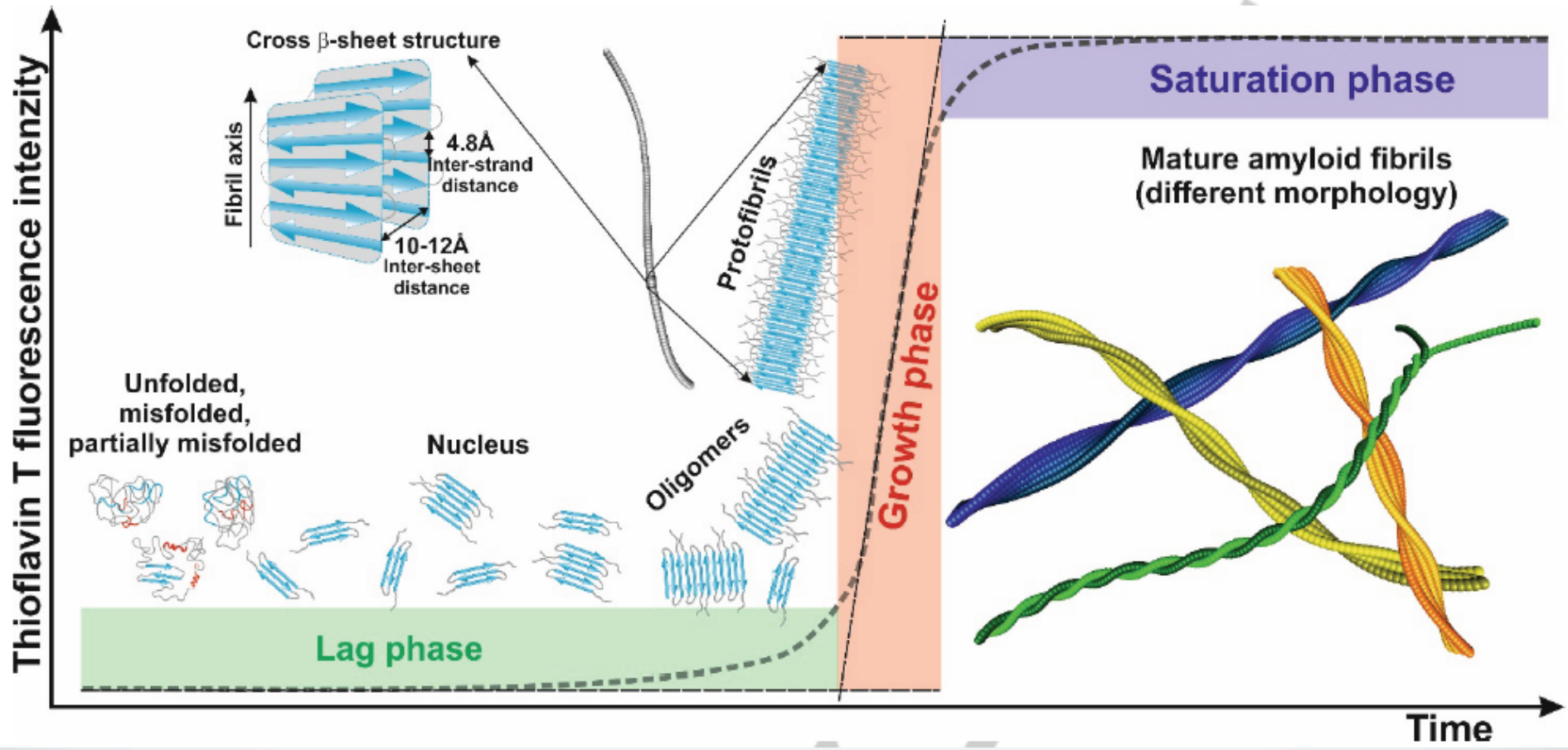
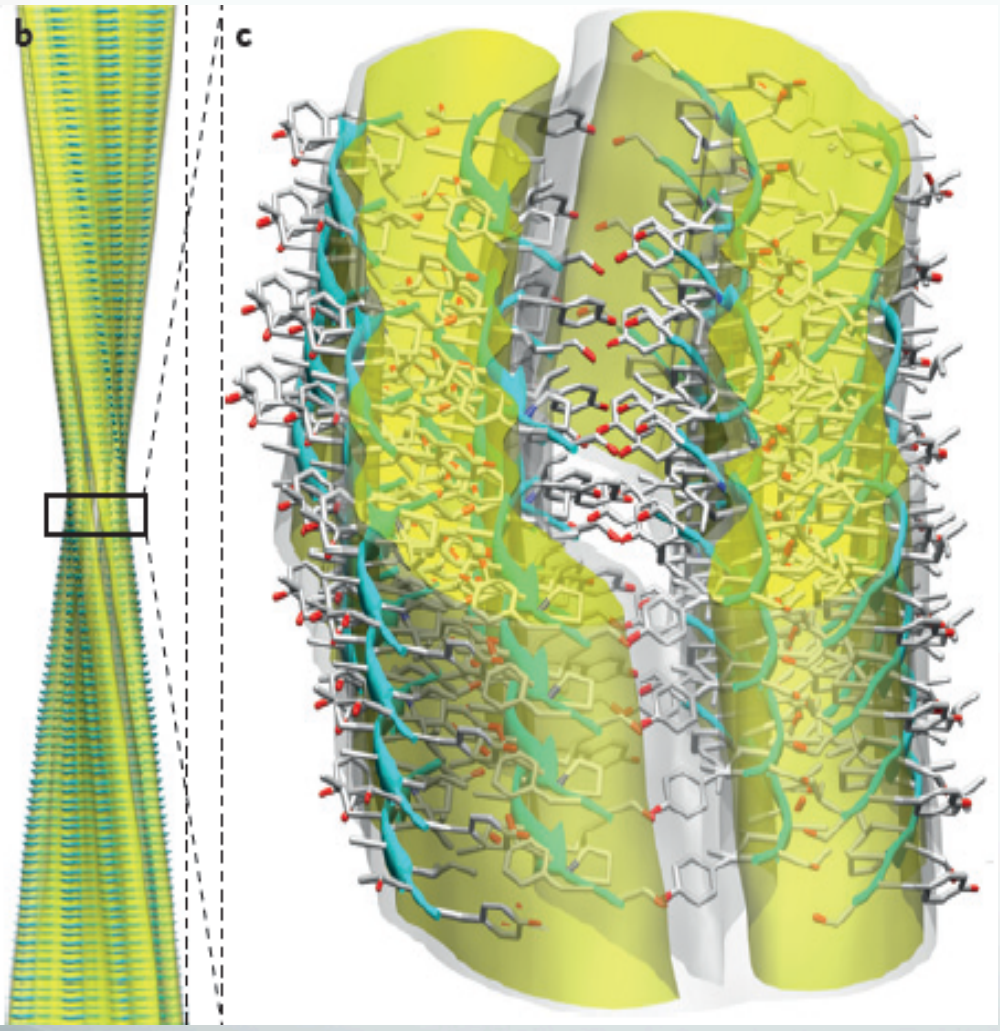


Figure 2. Self-assembly process of unfolded or misfolded peptide chains into mature amyloid fibrils. The process of ordered aggregation (amyloid fibrillization) has a typical sigmoidal shape containing three phases. The Lag phase correspond to the assembly of the peptide chains into small nuclei which grow by the intermolecular interactions-driven assembly into oligomers with β-sheet structures. In the second phase of the self-assembly process, the growth phase, nuclei and oligomers continue to interact together assembling into prefibrillar structures, which rapidly grow to form ordered fibrillar structures, the protofibrils. The third phase of self-assembly process, saturation phase, represents assembly of protofibrils into mature multi-stranded amyloid fibrils with different morphological structures and a high level of possible polymorphism.

Amyloid fibrils are typically composed of 2-6 protofilaments, which represent a substructure of fibrils of 2-5 nm in diameter.

The **protofilaments** can be associated laterally or twisted together forming mature amyloid fibrils which in images obtained by microscopy techniques such transmission electron microscopy (TEM) or atomic force microscopy (AFM) typically appear as unbranched filaments of a few nanometers in diameter and one micrometer or more in length



A detailed view (**part c**) shows the hierarchical organization of this amyloid fibril in which the three filaments that form the mature fibril illustrated here are in turn formed by pairs of **cross-β-protofilaments**, which are each composed of **pairs of β-sheets**.

The fibril surfaces are shown as electron density maps, and the constituent β-sheets are shown in a ribbon representation; oxygen, carbon, and nitrogen atoms are shown in **red**, **gray** and **blue**, respectively

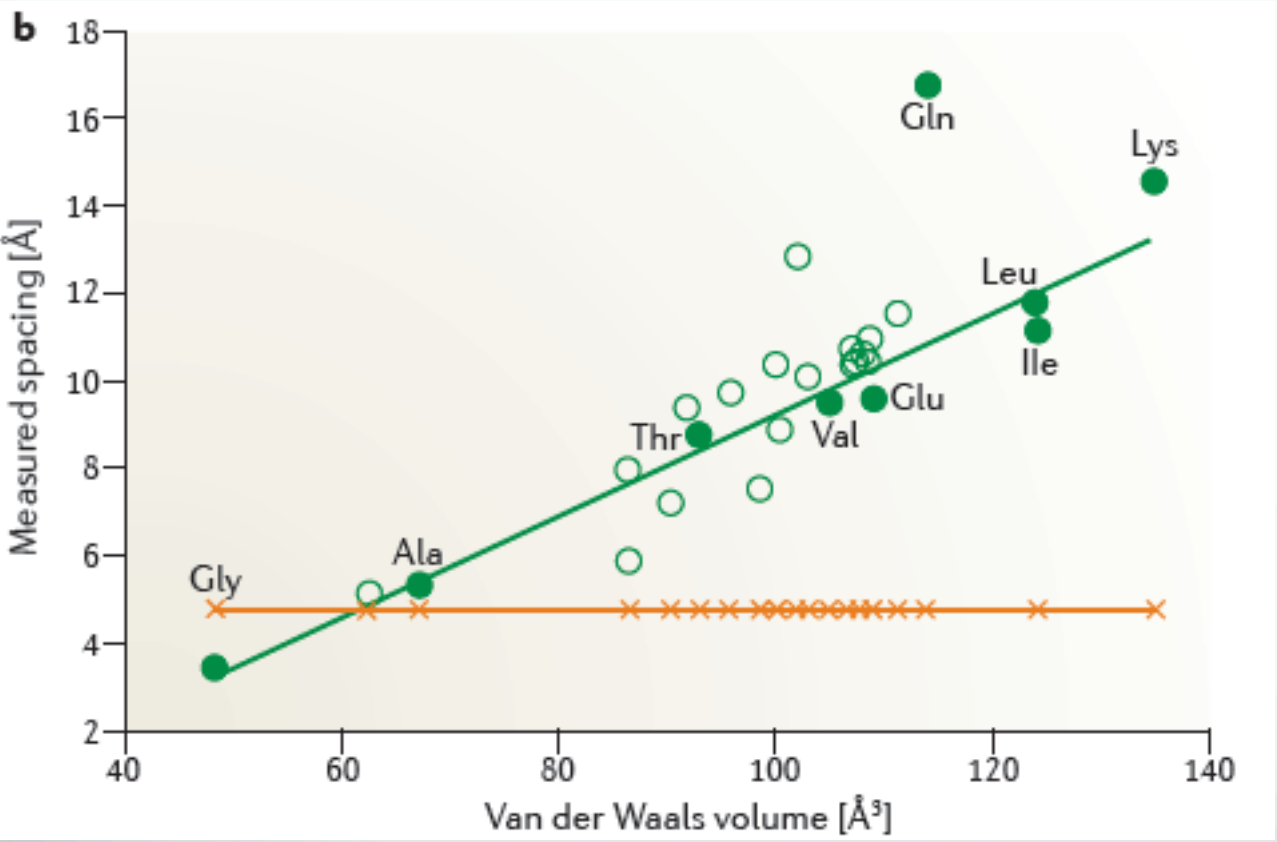
All these common features together with the evidence that the amyloid fibrils can be formed from a range of very different polypeptide sequences, have led to the suggestion that amyloid fibrils represents a **generic, widely accessible, stable structure of self-assembled proteins and peptides.**

This is in contrast with the native state of proteins, where the highly specific protein structure intimately depends on the amino acid sequence and where a complex protein folding process is required to establish the correctly folded structures.

This observation therefore raises the question of the nature of the interactions that stabilize the amyloid fibrils.

Experimentally it has been shown that the most important parameters encoding the intrinsic mechanical strength of amyloid fibrils are the **intermolecular forces between β-sheets and β-strands**, while **theoretical** work showed that a key point for the mechanical properties of amyloid fibrils is the **strong ordering of hydrogen bonds**, in which the hydrogen bonds between β-strands act as a chemical glue between the layers increasing the mechanical stability of fibrils

Physical basis for the amyloid structure



The common cross-β architecture of amyloid structures originates from the universal propensity of polypeptide chains to form backbone hydrogen bonding.

The lateral packing of such β-sheets, however, relies on specific patterns of interactions between side chains that depend on the amino acid sequences of the component proteins

It has been observed that **mixed fibrils**, composed of different polypeptide chains, **are rare**, and indeed contiguous domains in multidomain proteins tend to **differ in sequence to reduce** their propensity to aggregate.

An important question is **why the assembly of a large number of polypeptide chains** should generate this type of higher order structure. A series of theoretical studies has indicated how the amyloid characteristics **arise from the inherent properties** of polypeptide chains, including their persistence length (that is, the **local stiffness** of the polypeptide chain) and the **chirality** of the α-carbon atoms.

The greatest majority of information collected on the structural polymorphism of amyloid fibrils has been obtained on fibrils formed in vitro and thus it may not be directly and entirely applicable to pathological amyloids.

Therefore, it is very important to carry out comparable studies of amyloid polymorphism on fibrils obtained directly from in vivo samples

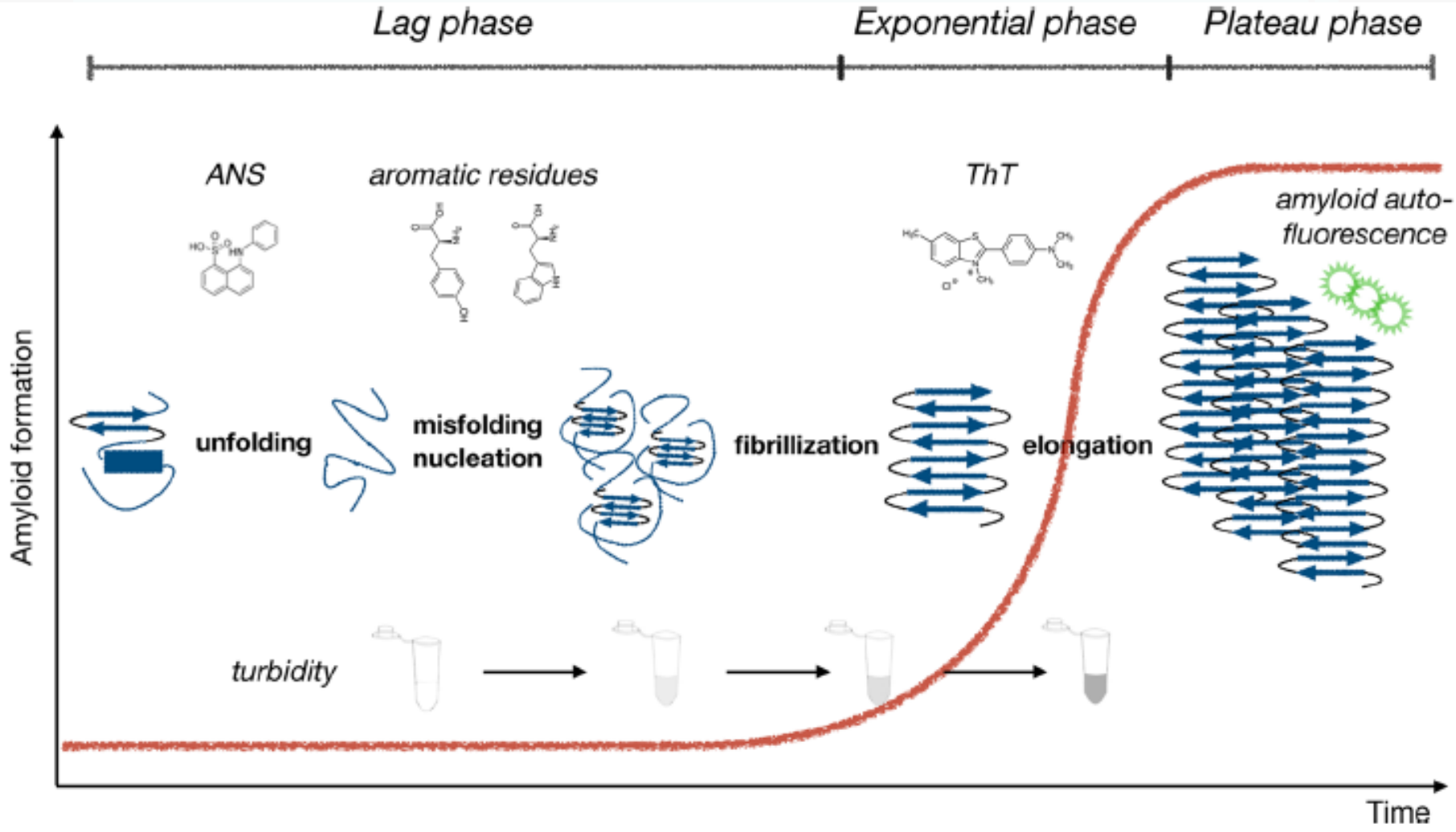


Fig. : Representation of different reporter strategies to monitor the process of protein aggregation in cell-free assays.

During the lag phase, the fluorescence of aromatic residues present in the polypeptide chain or extrinsic hydrophobicity-sensitive dies such as ANS are usually employed to readout the initial unfolding and nucleation events

During the exponential phase of fibril growth, ThT(Thioflavin T) and other amyloid-specific dyes are the most common tools to monitor in vitro aggregation.

The recently described autofluorescence intrinsic to amyloid structures constitutes an alternative strategy to detect the appearance of amyloid fibrils disposing of the use of extrinsic probes. Finally, measurement of the turbidity of the solution isa convenient approach when fluorescence could interfere with the assay readout

Stability of the amyloid state.

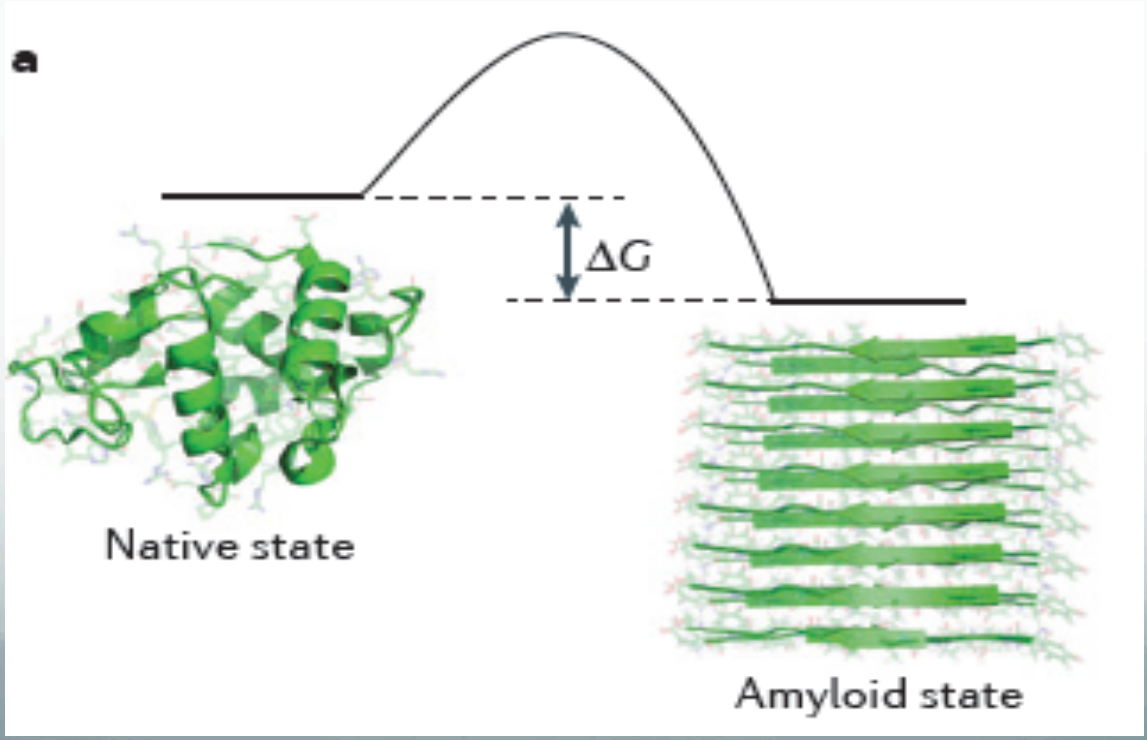
There has been increasing evidence, that the amyloid state might be **thermodynamically more stable** than the functional native states of many protein molecules even under physiological conditions. As the conversion of proteins from their soluble states to the amyloid form involves the formation of intermolecular contacts, the thermodynamic stability of the amyloid state is increasingly favoured at **higher concentrations**.

Amyloid fibrils are likely to become **thermodynamically unstable** compared to globular native structures for polypeptide chains of **more than ~150** residues because of the **topological constraints** that are associated with the packing of a long polypeptide chain into the fibril core.

Nature may have exploited this feature by **evolving proteins with 300–500 residues** on average to minimize the risk of amyloid formation.

In accordance with this view, the amyloid fibrils that are known to be associated with disease are all composed of relatively **short peptides or proteins**, or of **proteolytic fragments** of larger precursor proteins

These observations, therefore, have led to the **remarkable conclusion** that, at the concentrations present in living systems, the **native states may not always represent the absolute free energy minima** of the corresponding polypeptide chains — the native form of a protein could in some cases simply be a metastable monomeric (or functionally oligomeric) state that is **separated** from its polymeric amyloid form by **high kinetic barriers**

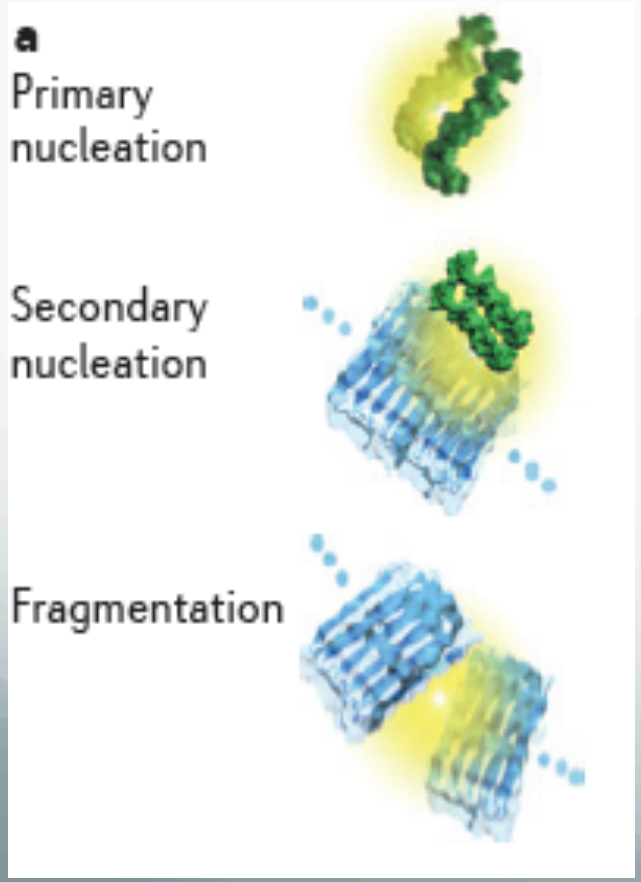


The kinetics of amyloid formation

The transition from soluble to fibrillar states.

The transition of a protein from its functional soluble state to the amyloid state is a **complex phenomenon** that results from the interplay between multiple precursor species, including **small assemblies** detectable by electron microscopy and AFM methods, as well as **protofibrillar structures**, which are generally much smaller than those of mature fibrils.

Many studies, in particular by **microscopic and mass spectrometric techniques** and by **single-molecule optical methods**, have revealed that the initial stages of the aggregation process involve the formation of a heterogeneous array of oligomeric species.



In some cases, including those of α-synuclein and yeast prions, these oligomers have been shown to undergo **slow transitions** from relatively disorganized species to more compact structures with a rudimentary cross-β structure and then are probably able to grow into fibrillar species.

Whereas **thermodynamics** describes whether or not a transition from one state to another is spontaneous and can occur without an external driving force**, kinetics** addresses the question of how fast such a transformation will take place.

USEFUL DEFINITIONS:

Templating

A phenomenon in which structured aggregates promote the conversion of soluble protein species into similar aggregates.

Primary nucleation
A nucleation process that takes place by the spontaneous assembly of monomeric species

Seeding
A phenomenon in nucleated growth processes by which nuclei of the aggregated phase promote the formation of larger aggregates.

Secondary nucleation
A process by which the formation of new nuclei in the aggregated phase is catalysed by existing aggregates.

Spreading
In the context of neurodegeneration, the spatial propagation of amyloid assemblies from cell to cell by a series of diffusion or transport mechanisms that are coupled with seeding or templating processes

As most of our detailed knowledge of the structures and of the thermodynamic and kinetic properties of amyloid fibrils has been obtained from *in vitro* studies, it is of great importance to relate these findings to the events that occur within living systems and hence to begin to explore the **molecular basis of the pathogenicity** of protein aggregates.

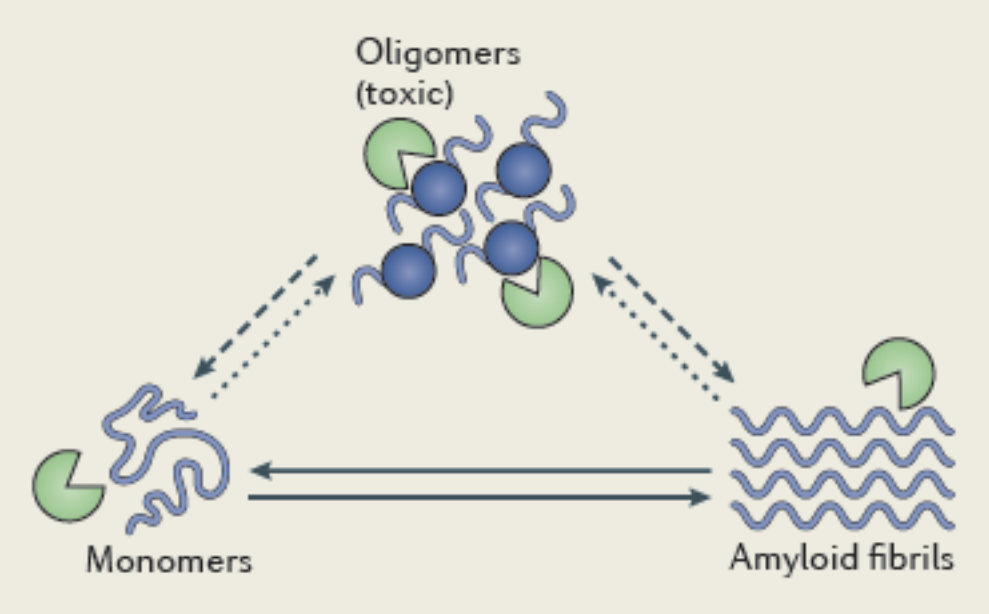
Studies of amyloid formation within simple model organisms, such as **fruitflies** and **nematode worms**, demonstrate that links can be made between the findings of detailed *in vitro* experiments and the events that take place *in vivo*. It is becoming increasingly possible to apply a wide range of biophysical approaches and imaging techniques to living systems to compare the events that occur in the different environments.

Despite the fact that it is clear that there is a close association between the appearance of amyloid deposits and the onset of pathological events in protein misfolding diseases, much remains to be understood about the specific mechanisms underlying these events.

- ✓ In the case of **systemic amyloidoses**, it seems likely that the primary cause of disease is simply the presence of large quantities, of amyloid deposits in vital organs, including the liver, spleen and kidney;
- ✓ In **neurodegenerative disorders**, by contrast, there are in many cases no detectable correlations between the overall quantity of fibrillar aggregates and the stage of disease advancement which suggests the possibility that these human disorders could be associated with misfolding events that induce cellular damage but that result in few persistent or readily detectable aggregates.

Indeed, a view has emerged over the last years that **pre-fibrillar species**, rather than mature amyloid fibrils, are likely to represent the **primary pathogenic agents** in non-systemic conditions, notably neurodegenerative diseases and other organ-specific conditions such as type II diabetes.

Indeed, increasing evidence suggests that the oligomeric assemblies that are almost universally observed as intermediates during the aggregation process are 'generically' damaging to cells.
This phenomenon has been observed both for proteins that are associated with disease and for those that are not linked to any known pathology.



The maintenance of protein homeostasis can be achieved by regulating the concentrations of the different states of all the proteins that are contained within a living system and by controlling the rates of conversion between them. To this end, it is particularly important to reduce the population of oligomeric species (pre-fibrillar species) by disrupting the processes of their formation (see the figure; dashed arrows) or by promoting the pathways of their removal (dotted arrows). These strategies can be implemented in various ways

Strategies for therapeutic intervention

Through the modulation of production processes (targeting synthesis or proteolysis), degradation (targeting the ubiquitin– proteasome and the autophagy systems) or stability (usually targeting the native state). For example, one can use the ability of antibodies, or artificially generated analogues such as affibodies, to selectively bind to the native states of aggregation-prone proteins, as binding generally results in increased stability and hence to a reduction in aggregation propensity.

In some cases, it might be possible to use antibody-based immunotherapy approaches to reduce the level of highly aggregation-prone species (such as amyloid-β) by **stimulating their clearance**

Antibodies and their analogues also offer other possibilities, one of which could be to **mimic the action of natural molecular chaperones** by targeting the aberrant misfolded species that give rise to cellular damage. If such 'artificial chaperones' can be developed and can be targeted to the appropriate location (for example, by enhancing their ability to cross the blood–brain barrier), then they could represent a highly effective therapy.

Furthermore, it is also becoming evident that **small molecules** can also function to suppress the early stages of protein aggregation, for example by binding to specific amyloidogenic species and by reducing the risk of nucleation and proliferation of pathogenic agents.