Structural integrity of β-sheet assembly

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Abstract
The folding of a protein from a sequence of amino acids to a well-defined tertiary structure is one of the most studied and enigmatic events to take place in biological systems. Relatively recently, it has been established that some proteins and peptides are able to take on conformations other than their native fold to form long fibres known as amyloid. In vivo, these are associated with misfolding diseases, such as Alzheimer’s disease, Type 2 diabetes and the myeloidoses. In vitro, peptide assembly leads to amyloid-like fibres that have high stability, resistance to degradation and high tensile strength. Remarkably, despite the lack of any obvious sequence similarity between these fibrillogenic proteins and peptides, all amyloid fibres share common structural characteristics and their underlying structure is known as ‘cross-β’. Nature is rich in β-sheet protein assemblies such as spider silk and other ‘useful’ amyloids such as curli from Escherichia coli, where the strength of fibrils is fundamental to their function.

Identifying sequence determinants of protein assembly
Numerous proteins and peptides are able to assemble to form β-sheet-rich amyloid fibrils associated with disease, from the 37-residue peptide IAPP (islet amyloid polypeptide) to the 253-residue prion protein (see [1,2] for reviews), although these proteins and peptides do not appear to share any obvious common sequence motifs. In addition, many non-disease-related sequences are able to assemble under optimal conditions to form amyloid-like fibrils, and it has been suggested that amyloid represents the lowest thermodynamic state achievable by a protein chain [1], and that any protein or peptide is capable of forming amyloid in vitro under particular environmental conditions [1]. This suggests that the backbone may be entirely responsible for the assembly process, and it is clear that the fibrils are stabilized by extensive hydrogen-bonding along the length of the β-sheets. However, the propensity for different sequences to form amyloid fibrils has been explored in detail, and a number of aggregation prediction algorithms have been produced [3,4] to try to predict the rate at which a particular sequence will assemble in an ordered β-sheet manner. In general, these algorithms assess the β-sheet propensity of residues, hydrophobicity and hydrophilicity, as well as the overall charge and charge distribution and the occurrence of aromatic residues [3–5]. Aromatic residues have been highlighted as contributing to the core structural stability of fibrils [5–7], although their presence is not necessary for assembly [8].

Designed short peptides are useful model systems for determining which residues are important in fibril formation [8]. Indeed, mutations in proteins are often the reason behind increased propensities to form amyloids. For example, an A53T substitution in the Parkinson’s disease-related peptide, α-synuclein, results in a peptide that forms fibrils at a much higher rate than the wild-type [9]. Studies reveal that a simple repeating peptide consisting of nine alternate valine and lysine residues forms amyloid-like fibres with a pronounced twist. Inserting a diproline moiety into the central region prevents twisting in the fibre structure [10]. It has also been noted that single amino acid substitutions can dramatically change not only the rate of fibril formation, but also the morphology of the resulting fibres [8] (Figures 1 and 2).

Understanding the assembly process
In general, the process of assembly begins with low-molecular-mass species that later transform into short thin assemblies called protofibrils that are visible by EM (electron microscopy) (Figure 1b). At later stages, mature fibrils form from protofilaments that associate both laterally and along the length of the fibril. Once amyloid has formed, it is able to act as a seed for the proliferation of more fibrils. This ability to convert normal soluble protein into the amyloid state is best exemplified by prion proteins that can act as inheritable infectious entities by a process of templated assembly [11].

The assembly of amyloid fibres is generally thought to occur initially via a lag phase where peptides assemble into a nucleus followed by a growth period (Figure 1). Fragmentation has also been pinpointed to help explain the observed kinetics of amyloid assembly [12,13]. Various biophysical tools are used to monitor assembly and report on different structural features of the fibrillization process. The dye thioflavin T undergoes a shift in its emission spectra from 342 to 442 nm upon binding to amyloid fibrils [14]. A plot detailing change in fluorescence intensity over time will generally show a sigmoidal shape corresponding to the slow initial nucleation process, followed by more rapid assembly.
Figure 1 | General kinetics for amyloid formation

(a) Fibril formation monitored by thioflavin T fluorescence. The classic sigmoidal shape represents a lag phase corresponding to the formation of a nucleus, followed by a period of rapid growth, finally reaching a stable plateau. (b) EM reveals the development of fibrils over time. (c) CD reveals the secondary-structural changes that accompany peptide assembly. The narrow line shows the spectrum from starting material (α-synuclein) giving a random coil signal, and the thick line shows the spectrum from fibrillar material consistent with an increase in β-sheet structure.

[15] (Figure 1a). Other fluorescence methods can be used to exploit the presence of the internal fluorophores tyrosine and tryptophan. A decrease in polarity of the environment around tryptophan causes a blue shift in the emission spectrum, a property which can be used to examine the extent of burial of the residue within fibres [16]. Tyrosine fluorescence, along with FRET (fluorescence resonance energy transfer), acrylamide quenching and fluorescence anisotropy, have been used previously to examine the role of the C-terminus of IAPP in fibrillation [17] and conformational changes in Aβ (amyloid β-peptide) during assembly [18].

CD (circular dichroism) is a standard technique for monitoring the change in conformation from natively folded or unfolded monomeric peptide to β-sheet-rich fibres [19,20] (Figure 1c). Secondary-structural details about the early unfolded or partially folded intermediates and their role in assembly can be drawn from CD data [21]. More recently, linear dichroism has been applied to the study of amyloid and has the additional benefit of providing information on the orientation of aromatic residues within the amyloid fibre [22].

Imaging techniques such as EM [23,24] and AFM (atomic force microscopy) [13] are able to capture images of the early species and can provide information on the growth of fibrils (Figure 1c). Recent work using TIRF (total internal fluorescence microscopy) [13,25] has shown that fibrillization of amyloid-forming peptides using thioflavin T labelling yields information about seeding events, growth and branching. Solution-state NMR and hydrogen–deuterium exchange can be applied at early stages of fibrillization to examine the dynamic interactions between monomers and early-stage soluble species [26]. The two techniques have been combined to probe the regions involved in the fibrillar core of Aβ fibrils and β2-microglobulin [27,28].

There is evidence to suggest that the first phase in amyloid formation is the assembly of oligomeric structures (this may be non-specific or specific, e.g. domain swapping). On the basis of a crystal structure of an amyloid fibril formed from the peptide GNNQQNY (a short fragment of the yeast prion protein Sup35), Nelson et al. [29] proposed a three-stage process for the formation of amyloid. In the first stage, hydrogen-bonded β-sheets form, followed by the less specific association of β-sheets via side-chain interactions to form a nucleus. The stability of the nucleus has been verified by Molecular Dynamics simulations on GNNQQNY, which confirmed that the overall stability of the fibril can be explained by interactions in the steric zipper [30]. Lastly, the fibril elongates from the nucleus [29,31]. The mechanism of amyloid growth has been examined using other systems such as the yeast prion protein Sup35 [32], where monomers are added to fibre ends after nucleation to propagate fibril extension. Furthermore, fibre fragmentation was taken into account to explain the observed growth kinetics. This observation has been supported by other research groups for different fibrillizing systems [12,33]. The growth of an F19P variant of the peptide Aβ-(1–40) has been followed in real time by SPR (surface plasmon resonance). It was shown to elongate by monomer addition in a reaction that is initially reversible, but leads to a more stable interaction over time [34]. Padrick and Miranker [35] characterized fibrillogenesis into a model called phase-mediated fibrillogenesis, where the elongation reaction is split into a concentration-independent lag phase and a concentration-dependent growth phase. So far, unlike structural inferences where the widely accepted conclusion is that the structure is cross-β, no single proposal has been adopted, and it is likely that there are many
different mechanisms by which oligomers and amyloid fibres form.

**Structure of the mature fibre**

Knowledge of the structure of amyloid fibrils is of interest both medically and for our understanding of protein folding and misfolding. Understanding at an atomic level can give insights into mechanisms of assembly and provide the ability to exploit any resulting rules. The structure of the amyloid fibril has been examined using EM and cryo-EM to reveal the protofilament composition of the fibres [24,36], suggesting that fibrils are composed of several protofilaments that twist around one another. Recent cryo-EM of Aβ-(1–40) [36] shows that fibrils in Alzheimer’s disease can be formed by the association of different numbers of protofilaments. Indeed, a wide variety of assembled structures have been observed, sometimes within the same sample [24,36] (Figure 2). Self-assembling peptides can form narrow smooth protofilaments, twisted ribbons and sheet-like structures and tubes. They can contain two or more protofilaments that wrap around one another to form a rope-like structure [24,37] and they can assemble into fibrous nanocrystals [6,8,29,31]. Exploring assembly conditions can lead to enrichment of a particular morphology and the structural form may be propagated by seeding with a pre-formed fibril of a particular structure [38]. It has been speculated that differences at the core structural level and at the macromolecular level could form the basis of disease ‘strains’, such as the prion-associated transmissible spongiform encephalopathies [11,38,39].

ssNMR (solid state NMR) has been used to probe the distance between labelled side chains within fibrils to enable conclusions to be drawn about the folding of the peptides within the protofilaments [40,41]. A model of Aβ-(1–40) suggests that the peptide folds into two β-strands with a β-bend that then forms the basis for two β-sheet ribbons [38,42]. ssNMR has also revealed that many peptides are able to form slightly different polymorphs that can be mixed in solution [41].

The inherent order and repetitive nature of amyloid fibrils enables the use of X-ray fibre diffraction as a tool to study the structure from the characteristic cross-β diffraction patterns with diffraction signals at 4.76 and 10–11 Å (1 Å = 0.1 nm) representing the hydrogen-bonding between β-strands and the intersheet spacings respectively (Figure 3). Diffraction patterns showing a high degree of orientation have enabled the structural modelling of several peptides using diffraction data [6,41–44] (Figure 3). The combination of fibre and electron diffraction led to a model of a designed amyloid-forming peptide in which aromatic groups associate between β-sheets via π–π stacking [6], again highlighting the potentially important role of aromatic residues in the core stability of fibrils [5]. Recently, a number of short peptides have been crystallized and categorized into eight classes of cross-β conformations described as the steric zippers [31]. However, X-ray fibre diffraction is the only technique capable of reporting long-range repetitive order within a fibre, and
Figure 3 | Cross-β diffraction pattern and structure
(a) The cross-β diffraction pattern observed for amyloid fibrils, taken from fibrils formed by Sup35 NM [47]. Arrows indicate positions of the cross-β reflections on the meridian at 4.7 Å (white) and on the equator at ~10 Å (black). (b) A simple repetitive structure composed of β-strands that run perpendicular to the fibre axis to form hydrogen-bonded β-sheets that associate via side-chain interactions. (c) A diffraction pattern calculated from the cross-β structure (b) using CLEARER [45] shows diffraction features similar to experimental data (shown in a).

variations between the diffraction patterns may be of interest. Diffraction patterns may be calculated from model structures to enable comparison between experimental and calculated data in order to evaluate structural features [41,45] (Figure 3).

Learning about assembly from non-pathogenic or ‘functional’ amyloid
The properties of amyloid fibrils that may contribute to their pathogenicity, such as their mechanical strength and ability to avoid degradation by the normal pathways, appear to have been exploited by living systems. The extracellular curli protein fibres expressed by Escherichia coli are involved in surface adhesion and aid the bacterium’s ability for host colonization [46]. They have been classified as amyloid on the basis of EM images, resistance to protease digestion, birefringence under cross-polarized light when stained with the dye Congo Red, a shift in thioflavin T fluorescence upon binding and computer modelling studies indicating a cross-β formation due to the glutamine- and asparagine-rich sequence [46]. Synthetic dragline spider silk shows similar X-ray fibre diffraction patterns to that of amyloid, suggesting a similar arrangement in at least part of the silk [47]. In contrast with the self-assembly of amyloid fibres in vitro, the production of silk by spiders is a tightly controlled process where various soluble proteins only become aggregated after passing through the spinning duct [48]. The mechanism by which this occurs is not fully understood, which is not surprising given the different types of threads that are produced, each with different properties designed for different functions [49].

Potential for amyloid-based bionanomaterials
With the wealth of knowledge on the assembly, structure and function of both disease-related and non-pathogenic amyloid, it has been possible to exploit its properties for technological purposes. A range of protein biomaterials have been created using a bottom up approach to their design based on first principles that include tissue scaffolds, hydrogels, nanotubes, biosensors and molecular motors [50,51]. Many examples focus on the ability of proteins to act as a support as they do in Nature, such as in collagen and the microtubule network in cells. Design of β-sheet-assembling peptides is hampered by the difficulties in controlling the system in comparison with the well-understood designed assembly of fibrous constructs based on coiled coils [52].

Amyloid-forming peptides have been designed to form nanowires for use in electronics industries. For example, gold nanoparticles have been covalently attached to cysteine residues within a variant of NM region (N-terminal and middle domains) of Sup35 and were bridged to form nanowires with a thickness of 80–200 nm [53]. Another example is the use of a diphenylalanine peptide that formed nanotubes ~100 nm wide and several micrometres long that are shown by FTIR (Fourier transform infrared) spectroscopy to have a β-sheet-like conformation. Ionic silver was added to the nanotubes and then reduced using sodium citrate. The peptide was then degraded with proteinase K to produce silver nanowires with a diameter of ~20 nm [54].

The possibility exists to create functional amyloid fibres by fusing with enzymes or other active moieties for use in bionanotechnological applications. Fusion proteins created from the N-terminus of the yeast prion Ure2p with various natively folded proteins assembled to form fibrils that retained the activity of the globular protein [55]. Similarly, fusing of the sequence for cytochrome b562 to an SH3 (Src homology 3) domain sequence resulted in amyloid fibrils that display the folded cytochrome domain on the surface and bind metalloporphyrins [56]. Systems like these enable control over enzyme activity, or, in the case of cytochrome, electron transfer via the self-assembly of fibrils with the additional benefit of the high stability of the amyloid core.
Conclusions
To exploit amyloidogenic peptides and their assembly fully, it is first essential to understand the dependence on sequence and assembly conditions, as well as the assembly process and resulting core structure. Amyloid fibrils have been shown to be highly stable and functionalizable, but this potentially valuable system must be controllable. Recent work incorporating switches that allow a trigger for the process of assembly and even disassembly may hold the key [57,58].

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