A Short History of Amyloid: from Abnormal Aggregation to Functional Assembly

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Amyloid fibrils are long protein fibrils, usually straight and unbranching (Fig. 1A), which have an underlying ordered β-sheet structure in which the β strands run at right angles to the fibril long axis. This structured core gives rise to a cross-β X-ray fibre diffraction pattern, with dominant reflections at ~4.7 Å on the meridian and ~10 Å on the equator of the pattern, and to diagnostic staining with the dye Congo red (Fig. 1B). This simple description of amyloid structure and character has held even as our understanding of the roles of amyloid fibrils in biology and disease has changed dramatically in recent years.

Extracellular, fibrillar amyloid deposits associated with human disease were first described by the pathologist Virchow in 1854 (1). The observation of apple-green birefringence from Congo red-stained deposits in tissue sections was considered pathognomonic for amyloidosis and different amyloid diseases were characterised by the nature of the component protein and the location of the deposits. When I joined Colin Blake’s group in the Laboratory of Molecular Biophysics in Oxford in late 1993, to start postdoctoral work on familial lysozyme amyloidosis, the study of disease-associated amyloid fibrils dominated the field (2). Clinician scientists such as Mark Pepys, Joel Buxbaum and Giampaolo Merlini led the way in diagnosis and treatment of systemic amyloid disease. At amyloid meetings, clinicians presented alongside basic scientists such as Jeff Kelly, Ron Wetzel, Dan Kirschner, Peter Lansbury and Paul Fraser, who were starting to apply biophysical methods to study disease-associated variant proteins in order to understand why proteins with a stable globular structure would self-assemble into an insoluble, fibrillar form (3). Colin Blake, as a pioneer protein crystallographer, had solved the three dimensional structures of both human lysozyme and transthyretin and he was keen to understand why and how variants of these two proteins formed amyloid fibrils and human disease. Louise Serpell was a DPhil student when I joined the laboratory and she initiated electron microscopy studies of transthyretin fibrils isolated from patients and also analysed detailed X-ray fibre diffraction patterns that she collected from Val30Met transthyretin amyloid fibrils, known to cause familial amyloidotic polyneuropathy (4). In collaboration with David Booth and Vittorio Bellotti, who were working with Mark Pepys in London, we started to study the process of fibril formation by two variant human lysozymes, identified as amyloidogenic in two families (5). Along the way we came to understand that all disease-associated fibrils had

Fig. 1.
A. Negatively stained transmission electron micrograph showing amyloid fibrils with typical long, relatively straight and unbranching morphology. These fibrils were formed from an amyloidogenic peptide derived from a fungal hydrophobin protein.
B. X-ray fibre diffraction pattern collected from the fibrils shown in part A, showing the strong inter-strand spacing at ~4.7 Å on the meridian, parallel to the fibril long axis, and the weaker and more diffuse inter-sheet spacing at ~10 Å on the equator of the pattern. Inset shows apple-green birefringence observed when amyloid is stained by Congo red.
C. Schematic representation of disease-associated fibril formation, where misfolding of the protein or polypeptide allows an amyloidogenic region (coloured green) to take part in intermolecular interactions, through hydrogen bonding, and to generate the β-sheet core of the amyloid fibrils. APP = amyloid precursor protein.
the common core cross-β structure, regardless of their native protein conformation and also recognised that disease-associated amyloid formation is essentially a protein misfolding process (Fig. 1C) (6).

As early as 1996, discussion between Colin Blake and Dan Kirschner at a Ciba Foundation Amyloid meeting in Portugal highlighted the fact that there were no known animal protein structures that displayed a cross-β structure. In β-keratin and most insect silks, the β-strands within the component β-sheets lie parallel to the fibril long axis. However, Dan Kirschner drew attention to the fact that the silk egg stalks produced by the lacewing *Chrysopa* had a cross-β-structure, described by Geddes in 1968 (7). This wasn’t recognised at the time for what it was, the first identification of a functional amyloid, but the work by Geddes and colleagues did underlie the development of models of human disease-associated amyloid fibrils (8).

With the realisation that disease-associated fibrils are often an undesirable end-point of protein misfolding, Chris Dobson, Sheena Radford and Carol Robinson brought protein-folding methodology and an understanding of chaperones to studies of fibril formation (5). It was at this time that Ivan Gujjarro, working with Chris Dobson in an effort to use NMR to study the folding pathway of an SH3 domain, found that it formed a gel in the bottom of his NMR tube when incubated at low pH for extended times. We realised that the gel contained amyloid fibrils with a cross-β structure, formed by misfolding of a domain never previously known to be associated with disease (9). This led to the idea that the amyloid fold is a generic protein fold, with (perhaps almost) all polypeptide sequences being able to form extended structures and to take part in the backbone, interchain hydrogen bonding (H-bonding) that forms the basis of the cross-β structure of amyloid fibrils (8).

Now amyloids were everywhere, produced from everything, under all conditions and in large quantities and these *in vitro* studies led to the recognition that oligomeric species, formed when misfolded monomers associated on the pathway to the formation of the long, straight, unbranching fibrils recognised as amyloid fibrils, were cytotoxic (10,11). Peptide fibril forming assays and mutational studies with model proteins were used to develop algorithms that could be used to predict and identify amyloidogenic sequences within proteins (12). Seeding was recognised as a key feature in the formation of prion amyloid deposits in the transmissible spongiform encephalopathies and Stan Prusiner’s work on prions led to him being awarded the Nobel Prize (13). High resolution work from David Eisenberg’s group, using microcrystals grown from short amyloidogenic peptides, underpinned our understanding of the cross-β structure of amyloid fibrils but highlighted the specific side-chain inter-digitation and unique β-sheet interfaces that may explain the homogeneous nature of most amyloid fibrils (14). This implied that the amyloid cross-β fold is more than a generic structure involving only H-bonding between amide and carbonyl groups of the polypeptide backbone. Cryo-electron microscopy and solid state NMR studies led by Helen Saibil and Rob Tycko gave us the first detailed information about the packing of amyloid protofibrils and protofilaments on a larger scale and in fibrils formed from full-length amyloidogenic proteins (15,16).

The understanding of protein (mis)folding intermediates and their interactions with chaperones grew with the increased understanding of the cellular machinery that acts to maintain proteostasis. Whereas disease-associated amyloid deposition had always been characterised in clinical samples as an extracellular phenomenon, there was a growing recognition of intracellular amyloid-structured aggregates. Sue Lindquist’s group connected yeast prions (structurally unrelated to mammalian Prion protein; PrP) and amyloids and it was shown that chaperones affected the maintenance of the prion state and amyloid formation (17). From 2000 onwards there has been a growing recognition that there is widespread application of the amyloid fibril structure for functional purposes in microorganisms. In addition to the Sup35 and Ure2p yeast prions, the fibrils formed from the fungal protein HET-s were shown to have an amyloid structure, with a highly-ordered β-solenoid structure (18). The Het-s element functions in the heterokaryon incompatibility system that leads to cell death. Fibrillar structures on the surface of *Streptomyces coelicor* spores and spores from filamentous fungi such as *Neurospora crassa*, long recognised and described by mycologists, were shown to have a robust amyloid nature, with the added property that they formed monolayers that were amphiathic and served to waterproof aerial bacterial and fungal structures (Fig. 2A) (19,20). It was demonstrated that bacterial curli fibres have an amyloid structure and that some of the natural adhesives produced by algae contain amyloid structures, adding mechanical strength to the substances (21). The list of natural, functional amyloids in microorganisms is likely to grow as there is recognition that the amyloid fold is applied in multiple settings and for multiple purposes: it can provide a stable superstructure (eg. lacewing egg stalk, curli fibrils), allow controlled self-assembly into a macromolecular form that generates or displays additional structural or functional properties (eg. amphipathic monolayer coatings on fungal spores) and can allow sequestration of peptides and proteins in stored, inactive or insoluble forms (eg. yeast prions or phenol-soluble modulins in *Staphylococcus aureus*) (22).

The first report of a mammalian functional amyloid came from Douglas Fowler and Jeff Kelly in 2005, when they published their finding that cytotoxic melanin biosynthetic intermediates are sequestered within melanosomes as a result of binding to PMel amyloid fibrils (23). In 2009, it was reported that certain peptide hormones were stored in an amyloid form (24). Not only did this show that unique amyloid fibrils could play important functional roles within mammalian systems, it demonstrated that the amyloid forming machinery was under tight control, presumably necessary to prevent the unwanted consequences and toxicity observed in amyloid-associated diseases. The list of recognized mammalian, functional amyloids continues to grow. In 2012, Hao Wu’s laboratory demonstrated that large
necroptosis-associated signalling complexes involving the kinases RIPK1 and RIPK3 are stabilised by hetero-oligomeric amyloid interactions formed by common RIP homotypic interaction motifs (RHIMs) (25). Sequence analysis indicates that these RHIMs are similar to the sequences that stabilize HET-s prion structures and which signal for heterokaryon incompatibility in fungi (26).

Many RNA-binding proteins, known to have (yeast) prion-like domains, have been shown to form aggregated structures within cells and in some cases these are linked to diseases such as amyotrophic lateral sclerosis. *In vitro* studies indicate that many of these proteins can form amyloid-like structures and some may form amyloid *in vivo* (27,28). These amyloids appear to be less stable than most microbial amyloids and also less stable than disease-associated fibrils found in humans. This may reflect their ability to undergo phase transitions and to allow the sequestering of proteins in functional but dynamic structures such as ribonucleoprotein granules (29).

Recently, my laboratory has found evidence that some viral RHIM-containing proteins are able to form amyloid fibrils (Fig. 2B, unpublished). While mammalian cells use functional amyloid structures to signal for cell death in response to viral infection, certain viruses express proteins that can form functional amyloid that may interfere with the host response and allow latent infection. The formation of hetero-oligomeric amyloid fibrils may be a distinctive feature of functional or biologically active amyloid, where different active domains are brought together through association of similar amyloidogenic motifs (Fig. 2C). Disease-associated amyloid fibrils isolated from human patients typically contain only a single protein component (30), although recent evidence indicates that Aβ and IAPP amyloid may be found together *in vivo* and that IAPP may seed Aβ amyloid formation (31). High-resolution studies of the amyloidogenic core of these hetero-amyloid fibrils will be required to determine how side-chain differences and sequence-specific recognition are both accommodated.

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**Fig. 2.**

A. Negatively stained transmission electron micrograph showing that amyloid fibrils formed by a hydrophobin protein associate laterally to form a stable protein layer that provides a functional, amphipathic coating on the surface of fungal spores in contact with air.

B. Viral proteins containing RHIM sequences can form amyloid fibrils. Here a fluorescent protein domain, substituting for the viral ribonucleotide reductase domain, is attached to the RHIM sequence from the M45 protein from murine cytomegalovirus. The fusion protein assembles spontaneously into amyloid fibrils, with the fluorescent partner domain displayed along the length of the assembled fibrils.

C. Schematic representation of two routes to the formation of functional amyloid fibrils. *Left panel:* Exposure or generation of an amyloidogenic motif (coloured green) by controlled conformational change or proteolysis can lead to fibril assembly. *Right panel:* Amyloidogenic motifs (coloured green) may be attached to separate, functional domains and assembly of the motif into the β-sheet amyloid structure can drive formation of fibrils decorated with the active domains.
Amyloid deposits are not always undesirable protein aggregates and not all undesirable protein aggregates are amyloid. A major challenge is to understand the mechanisms that control appropriate protein self-assembly for functional purposes and which allow turnover of biologically active amyloid.

References

1. Virchow, R. (1854) *Virchows Arch.* 6, 415-426