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Review

On the structural definition of amyloid fibrils and other polypeptide aggregates

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Abstract. Amyloid fibrils occur inside the human body, associated with ageing or a group of diseases that includes, amongst others, Alzheimer's disease, atherosclerosis and type II diabetes. Many natural polypeptide chains are able to form amyloid fibrils *in vivo* or *in vitro*, and this ability has been suggested to represent an inherent consequence of the chemical structure of the polypeptide chain. Recent literature has provided a wealth of information about the structure of aggregates, precipitates, amyloid fibrils and other types of fibrillar polypeptide assemblies. However, the biophysical meaning associated with these terms can differ considerably depending on the context of their usage. This overview presents a structural comparison of amyloid fibrils and other types of polypeptide assemblies and defines amyloid fibrils, based on structural considerations, as fibrillar polypeptide aggregates with a cross-β conformation.

Keywords. Amyloid, conformational disease, misfolding, prion, protein folding.

On the definition of 'amyloid fibrils'

'Amyloid' means starchlike. It is derived from the Greek word *amylon* (= starch) and was coined initially in a botanical context by Schleiden [1]. Virchow and others transferred the term 'amyloid' into medicine to describe human-pathogenic deposits that stain blue-violet with sulphuric acid and iodine solution [2]. However, these deposits do not primarily consist of polysaccharides. Instead, they were found to be proteinaceous [3]. Current definitions of 'amyloid fibrils' depend on the context of their usage. For the purpose of pathological diagnosis the Nomenclature Committee of the International Society of Amyloidosis has defined amyloid as 'extracellular depositions of protein fibrils with characteristic appearance in electron microscope, typical X-ray diffraction pattern, and affinity for Congo red with concomitant green birefringence' [4]. Moreover, pathological diagnosis bases some evidence for amyloid deposition not even on the fibrils themselves but rather on the presence of secondary components, such as serum amyloid P component [4, 5].

By contrast, scientists interested in revealing the molecular structure of amyloid fibrils and also the biophysical principles and mechanism of their formation require for their work a structure-based definition of amyloid fibrils. Such a definition assumes that amyloid fibrils represent an unique conformational arrangement of the polypeptide chain. Structure-based definitions must necessarily classify together what is structurally indistinguishable, while the precise conditions of tissue deposition are less important in this context. It follows that such definitions do not require an extracellular distribution and include also intracellular fibrils, such as polyglutamine fibrils or paired helical filaments [6]. Henceforth, amyloid fibrils are defined here as fibrillar polypeptide aggre-

gates with cross-β conformation. Whether or not a fibril is an amyloid fibril therefore depends on the presence of a cross-β structure. Normally, this type of structure is revealed with X-ray or electron diffraction. Other techniques may also be suitable for this purpose, but often these are associated with limitations. Fourier-transform infrared (FTIR) spectroscopy, for example, gives rise to an amide I′ maximum from 1610 to 1630 cm^{-1} that distinguishes the β-sheets of amyloid fibrils from those in most native globular proteins [7]. Although this method is also applicable to tissue sections [8], it does not discriminate amyloid fibrils from non-fibrillar aggregates [9, 10]. Binding of dyes, such as Congo red or thioflavin, can be used for amyloid diagnosis, but their specificity depends on solution and staining conditions [4, 11, 12], and examples of cross-reactions with non-fibrillar aggregates, globular proteins or non-proteinaceous materials have been documented [13–16]. While we do not yet fully understand what mediates binding of these dyes to non-amyloid structures, Congo red, for example, was found to intercalate between two aromatic ring systems in an insulin dimer [14], suggesting that interactions with aromatic residues could contribute to such affinities.

Amyloid fibrils *in vivo* and *in vitro*

The formation of amyloid fibrils inside the body represents a somatic and conformational deterioration of endogenous polypeptide chains. Amyloidosis (disease arising from amyloid formation) differs in this respect from viral or bacterial infections that critically involve the introduction of foreign nucleic acids or proteins. Amyloid formation can occur inside the brain and extracerebrally. It is associated with major diseases, including Alzheimer's disease, cardiac arrhythmias, atherosclerosis, rheumatoid arthritis and type II diabetes (Table 1). However, most cases of *in vivo* amyloid formation occur sporadically and as a somatic degeneration during the human ageing process. In other words, amyloid fibrils eventually accumulate in everybody after a certain age. 97 % of all humans aged over 50 years have acquired, in their aortic vessel wall, aortic medial amyloid [17], and virtually everybody aged over 80 years shows transthyretin (ATTR) amyloid deposits somewhere inside the body [5]. Amyloidosis can be caused by the massive deposition of amyloid fibrils so that the affected tissue is severely distorted. Such amyloid-inflicted physico-mechanical damage can destroy normal organ function and leads, in lattice corneal dystrophy, to blindness [18]. Other amyloidoses, notably AL amyloidosis, can even be lethal [12]. It has been estimated that more than 100 different human pathological conditions involve amyloid fibril formation [19]. Besides the physical effect of massive amyloid deposits, amyloid fibrils or certain prefibrillar aggregates are thought to be associated with cytotoxic activity and may lead to perforation of biological membranes. This pathogenicity mechanism is discussed most intensively for cerebral types of amyloid formation [20]. In contrast to this, there is also evidence that nature has utilised the basic construction principle of amyloid fibrils to build natural materials of high elasticity and persistence, such as the hydrophobin monolayer of fungal spores or fruiting bodies [21], or in the chorion of silkmoth eggs [22]. These data suggest that the presence of amyloid structures may not be detrimental *per se*.

Tissue-deposited amyloid fibrils consist of two components. The primary component is the actual fibrillar aggregate and consists of multiple copies of the same basic polypeptide sequence. Different types of amyloidosis are associated with different types of amyloid fibrils and amyloid fibril primary components; i.e. they differ by the sequence of the polypeptide chains forming the respective amyloid fibrils (see Table 1). In Alzheimer's disease, fibrils are derived from Aβ peptide, in type II diabetes from islet amyloid polypeptide and in rheumatoid arthritis from serum amyloid A protein [23]. Altogether, more than 25 non-homologous polypeptide sequences have been associated with amyloid fibril formation inside the human body [4, 12].

Secondary components are a chemically diverse group of substances, containing proteinaceous components, such as apolipoprotein E or serum amyloid P component, proteoglycans and lipids [5, 24, 25]. These secondary components are thought to decorate the fibrillar core of the deposit. Research in these secondary components is motivated by two main observations. First, secondary components are more or less conserved amongst different amyloid diseases, at least in the case of extracellular amyloid deposits. This suggests common principles of amyloid deposition, even if the diseases themselves are very different. Second, several of the amyloid fibril secondary components seem to be involved in amyloid pathogenicity. For example, serum amyloid P component is thought to protect amyloid deposits from body-own clearance mechanisms, thereby increasing the effective lifetime of these deposits [5]. Proteoglycans, on the other hand, promote aggregation reactions and fibril nucleation [24, 26].

Many natural and non-natural polypeptide chains can form amyloid fibrils *in vitro*. The first confirmed case of amyloid fibril formation *in vitro* was provided by a study using proteolytic fragments of Bence-Jones

Table 1. Some polypeptide sequences that form primary components of disease-associated amyloid deposits [4, 12].

Precursor polypeptide	Amyloid classification	Associated syndrome/disease (selected examples)
Aβ peptide	Aβ	Alzheimer's disease
Atrial natriuretic factor	AANF	cardiac arrhythmias
Apolipoprotein AI	AApoAI	atherosclerosis
Serum amyloid A protein	AA	rheumatoid arthritis
Cystatin C	ACys	cerebral amyloid angiopathy (Icelandic type)
Gelsolin	AGel	familial Finnish amyloidosis
Immunoglobulin light chain	AL	systemic AL amyloidosis
Islet amyloid polypeptide	AIAPP	diabetes mellitus, type II
Kerato-epithelin	AKer	lattice corneal dystrophy
Lysozyme	ALys	hereditary non-neuropathic systemic amyloidosis
Medin	AMed	aortic medial amyloid
β_2-Microglobulin	Aβ_2M	dialysis related amyloidosis
Prion protein	APrP	spongiform encephalopathies
Prolactin	APro	prolactinomas
Transthyretin	ATTR	familial amyloid polyneuropathy

protein [27]. However, even earlier examples of *in vitro* aggregation exist [28, 29], although the relevance of these data for amyloid structures was not known then. Amyloid fibrils may be formed *in vitro* from the same polypeptide precursors leading also to amyloid tissue deposits, such as from Aβ peptide or Bence-Jones protein (AL amyloidosis) [27]. However, even polypeptide sequences not known to produce any clinical forms of amyloidosis are capable of acting as amyloid fibril precursors *in vitro*. This was noted initially for amyloid fibrils formed from glucagon [30] and recently examples of these *in vitro* amyloid fibrils have multiplied very substantially [6, 31].

Initial observations that polypeptide chains can form fibrillar structures *in vitro* that are highly reminiscent of tissue-deposited amyloid fibrils sparked off a still lively debate as to whether or not these fibrils should be considered as amyloid fibrils or rather as 'amyloid-like' [4]. From a structural point of view, however, it is becoming increasingly clear that there exists no fundamental or systematic difference between these *in vitro* fibrils and tissue-deposited amyloid fibrils: both can be formed from the same polypeptide precursors; both possess, by negative stain transmission electron microscopy (TEM), similar overall structures and dimensions [27, 32]; both produce a characteristic cross-β diffraction pattern and give rise to green birefringence upon staining with Congo red [27]; both possess structural epitopes that enable binding of amyloid secondary components, such as serum amyloid P component [5]; both can stain with other dyes or tracers and produce a similar amide I region by infrared spectroscopy [8, 10].

Overall topology of mature amyloid fibrils

Initial evidence about the structure of tissue-embedded amyloid fibrils was provided with topo-optical reactions and polarising microscopy. These methods revealed substantial order within histological sections of amyloidotic deposits, along with an anisotropic orientation of the dye molecules [33–35]. TEM showed that this order is due to the presence of fibrils [36]. These fibrils can become several micrometers long (Fig. 1a) and have diameters ranging from 5 to 25 nm. Ultra-thin sections cut through tissue-extracted amyloid fibrils at an angle perpendicular to the main fibril axis showed that the fibrils possess a ring-like cross-section that consists of four to six density centres [37, 38]. Such an arrangement resembled the one of microtubules that consist of several protofilaments and enclose a hollow fibril core. A similar tubular protofilament arrangement was suggested, therefore, for amyloid fibrils as well [37]. The term 'protofilament' describes a linear row of protein molecules. Side-by-side interactions with other protofilaments construct a full fibril entity. Figure 1b shows structural models of amyloid fibrils consisting of two to four protofilaments.

Amyloid fibrils can show discernible periodicity, resulting in apparent constrictions ('crossovers') and dilatations of the fibril width in TEM images (Fig. 1a). Atomic force microscopy or platinum side shadowing show that this periodicity arises from a twisted and ribbonlike structure of the fibril (Fig. 1c). It is thought that amyloid fibrils generally possess a left-hand fibril twist [39–41] that arises from the inherent left-hand

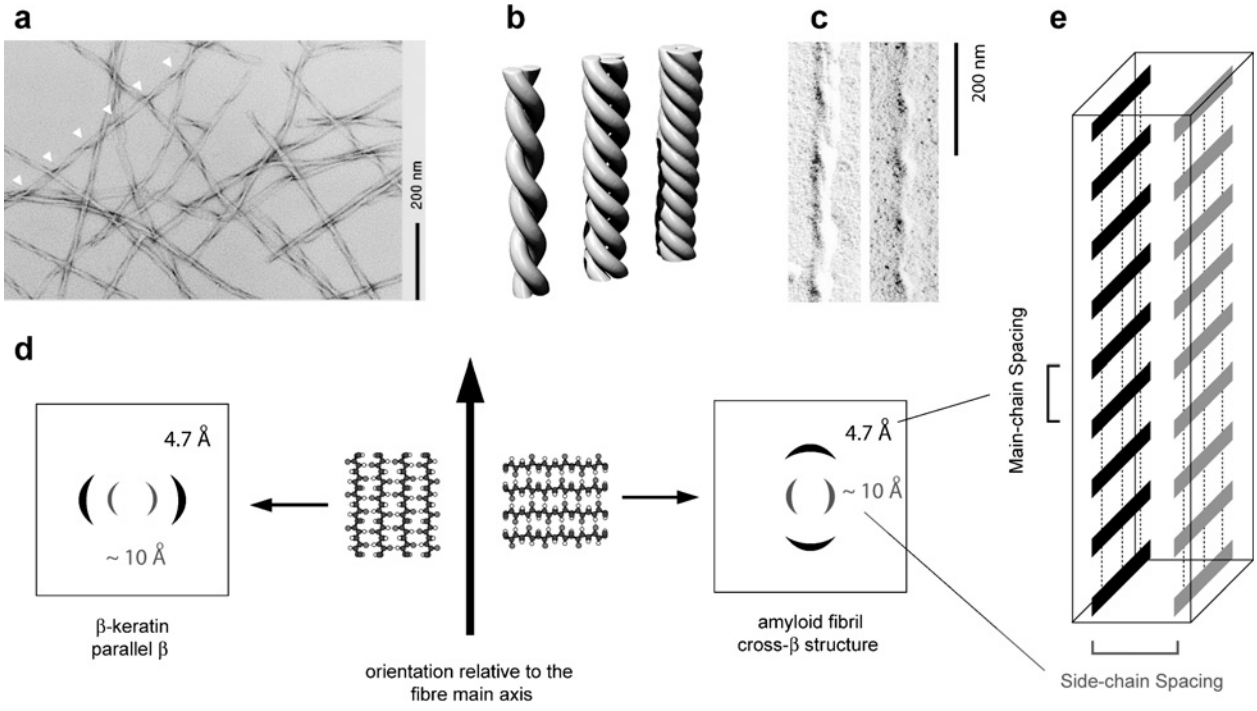

Figure 1. Structure of amyloid fibrils. (*a*) Electron micrograph of Aβ(1–40) amyloid fibrils showing crossovers (arrow heads) at regular distance. (*b*) Schematic representation of fibrils consisting of 2, 3 and 4 protofilaments. (*c*) Left-handed fibril chirality of Aβ(1–40) amyloid fibrils observed with transmission electron microscopy (TEM) after platinum side shadowing. (*d*) Schematic representation of the X-ray diffraction patterns of cross-β and parallel-β structures. (*e*) Protofilament structure interpretation based on X-ray diffraction (two laminated β-sheets, β-strands shown as black and grey bars; dotted lines indicate orientation of hydrogen bonds). This scheme does not show a twist. In (*b–e*), the fibril main axis is aligned in the vertical direction.

twist of natural β-sheets [42]. Single reports of fibrils with a right-hand chirality exist [43], but their molecular basis remains to be established. Furthermore, not all amyloid fibrils show a well-resolved fibril twist, either because the fibrils are intrinsically untwisted or because their twist is too tight to be discernible with these techniques. Amyloid fibrils can have appreciably different morphology, even when formed from the same polypeptide precursor and under the same incubation conditions [39, 40]. These morphological differences manifest themselves in differences in the fibril width and in the distance of adjacent crossovers. Different morphologies can arise from different numbers of protofilaments [40] or from different arrangements of these protofibrils.

The very large molecular mass of amyloid fibrils and also their inability to crystallise readily has hampered analysis by X-ray crystallography and standard solution-state nuclear magnetic resonance (NMR). Knowledge about the fibril architecture comes, therefore, from a range of different techniques, each describing a specific property of the fibril structure. These techniques include electron and atomic force microscopy [39], infrared spectroscopy [7], X-ray diffraction [44, 45] and electron diffraction [46]. Evidence for the location of the β-strands can be provided by mutagenesis, which reveals regions that are particularly susceptible to residual exchange, in particular when inserting proline that is highly unfavourable to β-sheet structure [47–49]. Additional techniques include hydrogen/deuterium exchange [50], electron spin resonance [51], solid-state NMR [52] and cryo TEM [40, 41, 53–55]. Detailed information has been provided with solid-state NMR spectroscopy or X-ray crystallography for a protofilament formed from a fragment of β_2-microglobulin [56] as well as for microcrystals in which peptide fragments of transthyretin or *Saccharomycies cerevisiae* sup35 protein have adopted crystallographic contacts closely reminiscent of amyloid fibrils [57, 58].

The cross-β sheet structure

Initial evidence about the structure of the polypeptide chains within amyloid fibrils was provided by X-ray diffraction [44]. This technique leads to a characteristic pattern of reflections (Fig. 1d), and it is now known that all amyloid fibrils give rise to such a diffraction pattern [59]. A cross-β diffraction pattern consists primarily of a conserved meridional 4.6–4.8 Å spacing and a more variable equatorial spacing,

corresponding to 5–12 Å [59, 60]. The molecular distances associated with these spacings and their orientations are interpreted in terms of a cross-β structure, which means that the β-strands of these sheets are oriented transversely to (and the hydrogen bonds in parallel to) the main fibril axis (Fig. 1d, e). This type of diffraction pattern was described initially in 1935 [29] and has been termed ever since the 'cross-β' pattern in order to distinguish it from the parallel-β case, where the β-strands are oriented parallel to the main fibril axis (Fig. 3d). Parallel-β structures occur in β-keratin or *Bombyx mori* silk.

Since the 4.6–4.8 Å spacing represents the distance between two hydrogen-bonded strands, it is referred to as the 'main chain spacing'. This spacing depends primarily on the invariant geometry of the polypeptide backbone and differs only marginally for different amyloid fibrils. By contrast, the 5–12 Å reflection, or 'side chain spacing', represents the packing distance of two juxtaposed β-sheets. Since the geometry of β-sheets is such that the side chains protrude orthogonally from the plane of the sheet [61], this distance is much more variable and depends, in a predictable manner, on the average van der Waals volume of the amino acid residues and on the polypeptide sequence involved in amyloid fibril formation [60, 62]. A combination of X-ray diffraction and cryo TEM data provided a model for the overall structure of amyloid fibrils formed *in vitro* from SH3-domains [31, 54].

The β-sheets of amyloid fibrils differ in several aspects from the ones of native globular proteins. This conformational difference can be revealed with FTIR spectroscopy as well as with conformation-sensitive anti-amyloid antibodies and amyloid secondary components. Conformation-sensitive anti-amyloid antibodies, such as WO1 and WO2 [63], or secondary components, such as serum amyloid P component [5], bind most, if not all, amyloid fibrils, although they do not interact with native β-sheet proteins. FTIR spectra of native transthyretin, a protein with more than 50 % native β-sheet structure, show a broad amide I′ band with a maximum at 1630 cm^{-1} (Fig. 2a). TTR amyloid fibrils lead to a much narrower amide I′ band that possesses a maximum at 1615 cm^{-1} and an additional low-intensity peak at 1684 cm^{-1} (Fig. 2b). Similar observations were made also for ubiquitin [7]. A literature survey of amide I′ data recorded on amyloid fibrils and native β-sheet proteins revealed that amyloid fibrils have amide I' maxima in the range from 1611 cm^{-1} to 1630 cm^{-1}, whereas native β-sheet proteins have amide I′ peaks clustering between 1630 cm^{-1} and 1643 cm^{-1} [7]. Examination of histological sections through amyloidotic tissue with FTIR microscopy confirm these infrared properties for amyloid tissue deposits [8]. Whereas the molecular basis of these differences is not fully clear, there is no obvious relationship to the presence of parallel or antiparallel structure. Instead, the infrared differences were suggested to result from a lower degree of twisting of the β-sheets in amyloid fibrils [7].

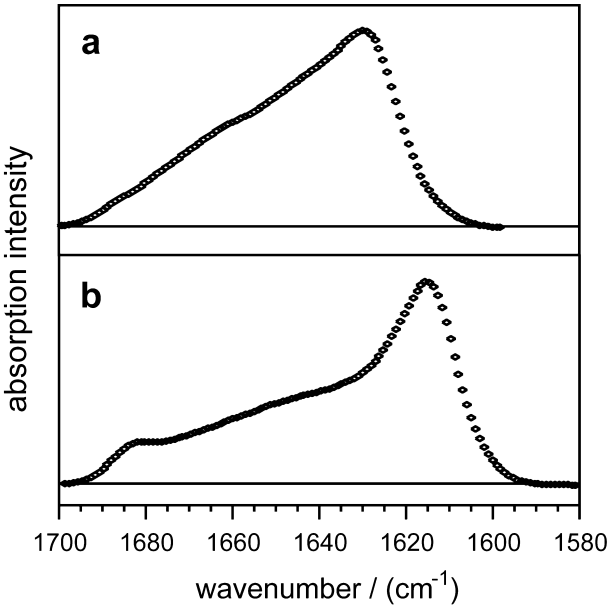

Figure 2. Infrared spectra of fibrillar and native transthyretin. Amide I′ region from the FTIR spectrum of native transthyretin (*a*) and of transthyretin amyloid fibrils (*b*). Redrawn after Zandomeneghi et al. (2004) [7].

Another question about amyloid fibril structures concerns the relative orientation of the β-strands, i.e. whether the β-sheets are parallel, antiparallel or mixed. Depending on the experimental technique and its interpretation, different conclusions were reached. X-ray diffraction, for example, lacks generally the 9.4 Å spacing expected for antiparallel structures [64], while infrared spectra of amyloid fibrils have a shape previously thought to be indicative of an antiparallel structure [9, 10]. Solid-state NMR spectroscopy suggests that some amyloid fibrils possess parallel β-sheets, while others are antiparallel [52, 56]. Parallel β-sheet structures were reported for the microcrystals of sup35 protein fragments [58].

Structure of 'protofibrils' and other fibrillar polypeptide aggregates

The overall fibril characteristics described above relate primarily to mature amyloid fibrils, the end products of amyloid fibril formation. These fibrils can be preceded, however, by metastable precursor ag-

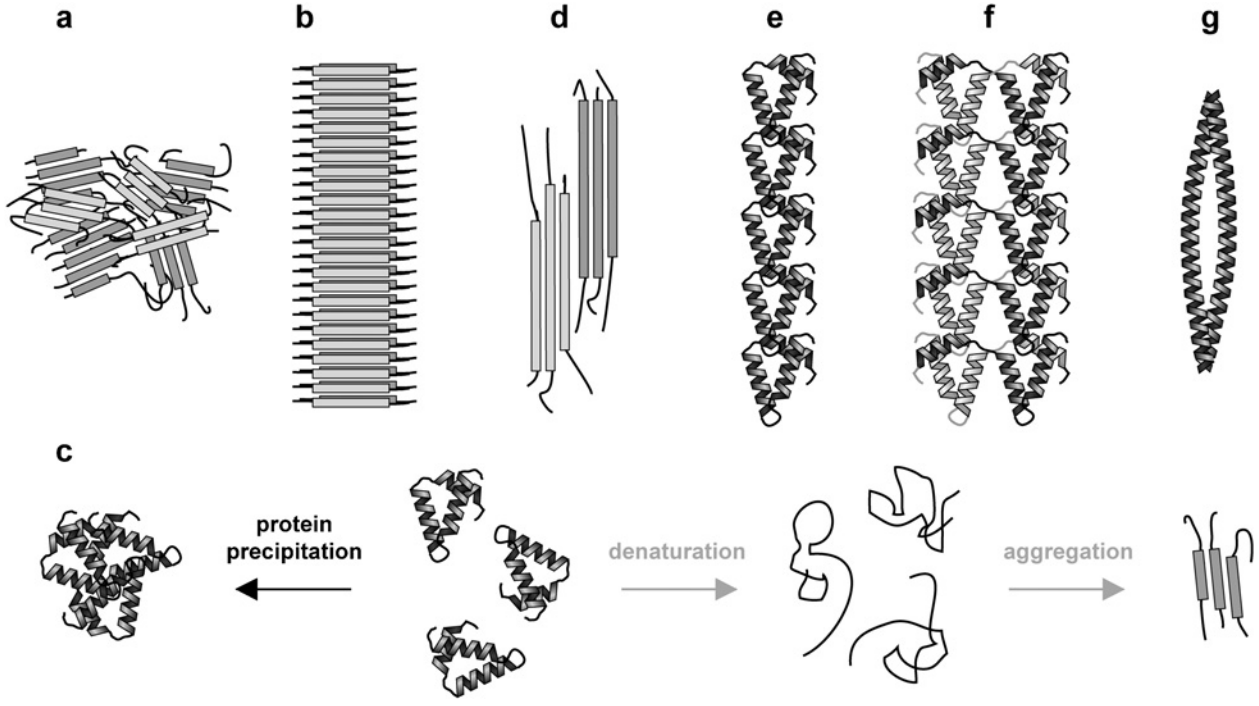

Figure 3. Comparison of amyloid fibrils with various other types of polypeptide assemblies. Structural representation of non-fibrillar aggregates (*a*) and amyloid fibrils (*b*). β-Strands are shown as grey bars. (*c*) Aggregation and protein precipitation of a hypothetical α-helical protein. Aggregation requires denaturation (= unfolding) of the globular protein structure. The resulting aggregates are constructed of β-sheets, while the protein precipitates consist, in this case, of α-helices. Fibrils (*d*) with parallel β-structure, (*e*) from a hypothetical globular protein, (*f*) from a globular protein with domain swapping and (*g*) from helical polypeptides, here a schematic representation of a coiled-coil-like arrangement. Fibril axes in (*b*) and (*d–g*) run vertically.

gregates, termed 'protofibrils' (as opposed to 'protofilaments'). Protofibrils were described initially to possess a thinner diameter (2–5 nm) than mature fibrils, to be shorter, curlier and more irregular ('wormlike') in their overall structure than mature fibrils [65]. Besides these linear aggregates, the term 'protofibril' has been applied also for aggregates of different structure, for example for spherical and annular assemblies [65, 66]. By contrast, mature fibrils are often associated with a more straight, long and regular appearance. Usually, they are thought to consist of more than one protofilament [65]. Numerous laboratories coined other names to define specific types of non-fibrillar aggregates or aggregation intermediates, such as 'soluble oligomers', 'oligomeric Aβ ligands' or 'ADDLs' and others [67, 68].

While amyloid diagnosis with clinical tissue samples involves an extracellular fibril deposition, they are several intracellular fibrils known to possess significant structural similarities to these extracellular amyloid fibrils. Examples hereof are the 'paired helical filaments' formed in Alzheimer's disease from tau protein [69, 70], polyglutamine fibrils in Huntington's disease [71], also known as 'polar zippers' [72], or the Lewy body associated α-synuclein fibrils found in Parkinson's disease [20]. Finally, there are the 'proteinaceous infectious particles' or 'prions' [73]. How these relate to amyloid fibrils is not fully understood. Although patients suffering from prion diseases are often associated with amyloid deposition [73], and although considerable other data suggest that prions are polypeptide aggregates according to the above definitions, prions are primarily defined by a specific infectious activity, and as long as the molecular nature and the structure of prions have not been clarified in more detail, it is difficult to compare them with amyloid fibril structures. A recent study suggests, however, that prion pathogenicity is associated with small, non-fibrillar aggregates [74].

On the structural definition of aggregates

Comparison of the terms 'aggregates' and 'amyloid fibrils' is complicated by the fact that the term 'aggregate' is used throughout the literature in very heterogeneous ways. In the present context, the term 'polypeptide aggregates' assumes the presence of an intermolecular, specific and usually abnormal type of β-sheet structure. These β-sheet structures share several of the structural characteristics detailed above for amyloid fibrils, such as an amide I′

maximum between 1610 and 1630 cm^{-1} [7, 9, 10], an X-ray diffraction pattern with a strong reflection at 4.6–4.8 Å and a second (side chain) reflection of more variable position [75, 76], the ability to bind Congo red or thioflavin dyes [16]. Furthermore, the way in which they respond to single-site amino-acid replacements in their β-sheet structure is also very similar as for amyloid fibrils [16, 77, 78].

While these features are very similar to amyloid fibrils, the latter are characterised, in addition, by their linear structure. This structure is visible by TEM or atomic force microscopy or by the anisotropy of the X-ray diffraction pattern. Non-fibrillar aggregates do not possess a linear overall structure, and their X-ray diffraction patterns contain concentric (isotropic) diffraction rings [28, 75]. However, the above data show that there are no fundamental differences between amyloid fibrils and these non-fibrillar aggregates. Both are constructed from the same type of β-sheet conformation, but these structural elements are only nascently present in non-fibrillar aggregates and do not show the very high order of amyloid fibrils (Fig. 3a). Therefore, amyloid fibrils are defined here as a specific form of aggregates, namely as aggregates where these β-sheets are ordered to an extent so that they give rise to a fibrillar overall organisation (Fig. 3b).

Due to these close structural similarities, it is not surprising that non-fibrillar aggregates can be found as pre-fibrillar aggregates during fibril assembly and to act as nuclei during fibril formation [76, 79, 80]. Note that the term 'non-fibrillar aggregate' is often synonymous with the frequently used expression 'amorphous aggregate'. The latter term is almost always based only on TEM or atomic force microscopy analysis and describes a non-fibrillar and often also very heterogeneous overall structure. In most cases, however, it is not established where these structures are truely amorphous and whether the polypeptides constructing these assemblies possess a random-coil-like, i.e. amorphous, structure (if such assemblies exist at all). TEM and atomic force microscopy provide no information about this question. Moreover, in many examples that FTIR spectroscopy, X-ray diffraction, dye binding and other techniques have shown, even these non-fibrillar and apparently non-regular aggregates are significantly structured [9, 10, 16, 75, 76]. Therefore, the term 'amorphous' is often misleading and should better be avoided in this context. By this definition non-fibrillar aggregates also include aggregates formed after thermal denaturation and some inclusion bodies [9, 10].

Another common characteristic of amyloid fibrils and non-fibrillar aggregates is the fact that their structure can be fundamentally different from the one adopted by the same polypeptide chain during native protein folding, the state of a globular protein. For example, myoglobin can form non-fibrillar aggregates or amyloid fibrils that are, of course, characteristically rich in β-sheet structure [81, 82]. However, native myoglobin is an entirely α-helical protein that lacks any elements of β-sheet structure. Since all aggregates are characterised by intermolecular backbone hydrogen bonds and an intermolecular β-sheet structure, proteins such as myoglobin can form aggregates or amyloid fibrils only if native protein folding does not prevent the intermolecular association of solvent-exposed and aggregation-competent main chains (Fig. 3c). Amyloid fibrils and non-fibrillar aggregates are formed, therefore, from relatively similar precursors, namely from partially, if not entirely, unfolded polypeptide chains [75, 83, 84]. Their formation can be promoted by mutation or denaturing conditions so that the globular protein state becomes unfavourable [83, 84]. Furthermore, polypeptide chains that are intrinsically unable to fold into a globular protein, such as Aβ peptide or polyamino acids [60, 85], are particularly susceptible to aggregation. It follows from the high order in amyloid fibrils that fibril formation requires more ideal interactions and that steric repulsions or other structural imperfections are much more difficult to tolerate than in non-fibrillar aggregation. Consistent with this idea myoglobin was found to form non-fibrillar aggregates under conditions where unfolding is partial and where part of the native-like α-helical structure is retained, while amyloid fibrils occur under conditions correlating with a more full unfolding reaction [75]. In cases where the native structure of a protein is already rich in β-sheets, however, there is evidence that amyloid fibril precursors may retain elements of a native-like β-sheet structure [84, 86–88]. Nevertheless, FTIR spectroscopy and binding studies with conformation-sensitive antibodies or some secondary components suggest that even β-sheet proteins must have undergone a more or less substantial structural reorganisation in order to form a fibril [5, 7, 63].

Structure of protein precipitates and globular protein fibrils

While polypeptide aggregates depend on the formation of a specific type of β-sheet structure, 'protein precipitates', as long as these represent simple cases of protein insolubility, do not require denaturation or substantial structural rearrangements (Fig. 3c). Well-known forms of protein precipitates are protein crystals [89] in which the native protein structure is retained. Absence of substantial conformational re-

arrangements upon precipitation can be demonstrated, for example with FTIR spectroscopy (similarity of the amide I region of the soluble protein and precipitate) [10]. Protein precipitation occurs when native proteins are salted out or exposed to their isoelectric points. However, it should be mentioned that there may be no general way to separate between aggregation and precipitation and more intermediate cases may well exist.

Amongst the fibrillar polypeptide structures are also cases that do not require any loss of native structural elements. These are the globular protein fibrils (Fig. 3e) that represent mere assemblies of globular proteins. This does not exclude than their assembly can be associated with small structural adjustments in otherwise flexible regions of the protein or within loop structures. Lithostatine fibrils are one example of such fibrils. These fibrils do not bind Congo red and retain, within the fibril, a native β-sheet structure, as evidenced by FTIR spectroscopy [90]. Other examples of such assemblies are microtubules, actin filaments and bacterial flagella. Moreover, there are assembly reactions which involve primarily a domain-swapping event (Figure 3f), such as in the α_1-antitrypsin deficiency [91, 92], or fibrils consisting of helical polypeptide chains (Fig. 3 g), such as collagen or intermediate filaments [93].

Structure of amyloid fibrils from Alzheimer's Aβ(1–40) peptide

Perhaps the most intensively studied types of amyloid fibrils are those formed from Alzheimer's Aβ peptide. Aβ peptide occurs inside the brain in isoforms of different lengths. Their size ranges from 37 to 43 amino acids. While the 42-residue isoform is significantly enhanced in Alzheimer patients, the 40-residue isoform Aβ(1–40) is generally the most abundant one, both in the normal brain as well as in Alzheimer patients [94]. This section focuses exclusively on the Aβ(1–40), while overviews on structural data acquired with various of the other Aβ isoforms and Aβ fragments can be found elsewhere [52, 95]. Numerous techniques have been employed to study the structure of Aβ(1–40) amyloid fibrils, for example negative stain TEM [39] and cryo TEM [41], atomic force microscopy [80], solid-state NMR spectroscopy [52, 96–98], hydrogen/deuterium exchange studies coupled with mass spectrometry [99] or NMR [50], site-directed mutagenesis [47–49] and proteolysis [100].

The fibrils formed from this peptide were shown to occur with significantly different morphologies [39], even within the same sample. One of the first structural models suggested for fibrillar Aβ peptide encompasses a U-shaped conformation where two β-strands are aligned side by side [101]. Support for this arrangement is provided by observations that a lactam-enforced alignment of two putative β-strand regions of Aβ promotes fibril nucleation [102] and solid-state NMR spectroscopy [96]. The latter technique suggested, in addition, the presence of an unfolded and random-coil-like N-terminus, corresponding to residues 1–8 (Fig. 4a). Assuming an unfolded N-terminus is also consistent with results from limited proteolysis [100].

An important feature of the peptide model derived from solid-state NMR spectroscopy is that the two strands do not form a common β-sheet, i.e. they do not interact through intramolecular backbone hydrogen bonds (as would be the case in a β-turn). Instead, the peptide forms a β-arch structure [103] where intramolecular interactions of the two β-strands are formed by their side chains (Fig. 4a). This conformation enables the formation of intermolecular backbone hydrogen bonds between juxtaposed peptide molecules, and ultimately the formation of a cross-β-sheet structure (Fig. 4b). Different fibril morphologies can be associated with different incubation conditions and peptide structures [97], and different studies or techniques assign the β-strands and the β-arch region to slightly different residues. Some studies even imply the presence of three instead of two β-sheets. Solid-state NMR spectroscopy suggests that residues 12–24 and 30–40 are present in a β-strand conformation [96]. Hydrogen/deuterium exchange experiments coupled with solution-state NMR spectroscopy shows that regions 17–24 and 28–35 are most highly protected from exchange [50]. Proline scanning mutagenesis reveals three regions (15–21, 24–28 and 31–36) that show the most significant response upon mutagenesis [47]. Approximately the same regions also show the strongest response in an alanine scan [48], while cysteine-scanning mutagenensis yields a more complex picture [49].

Solid-state NMR spectroscopy has provided information about the local quaternary structure and the relative orientation of adjacent peptide molecules [52, 98]. Cryo TEM has been used to explore the global fibril structure [41]. The advantage of the latter method is that it is a single-particle technique and enables analysis of a single-fibril morphology irrespective of the potential presence of other aggregates. The fibril morphology analysed with cryo TEM possesses a pitch of 270 nm and a fibril diameter of 20.5 nm (Fig. 4c). There is no hollow core or ringlike cross-section that would compare with the structure of some other amyloid fibrils [38, 54]. The investigated Aβ(1–40) fibril is ribbonlike and polar (Fig. 4c). Its cross-section resembles in shape the letter S (Fig. 4d).

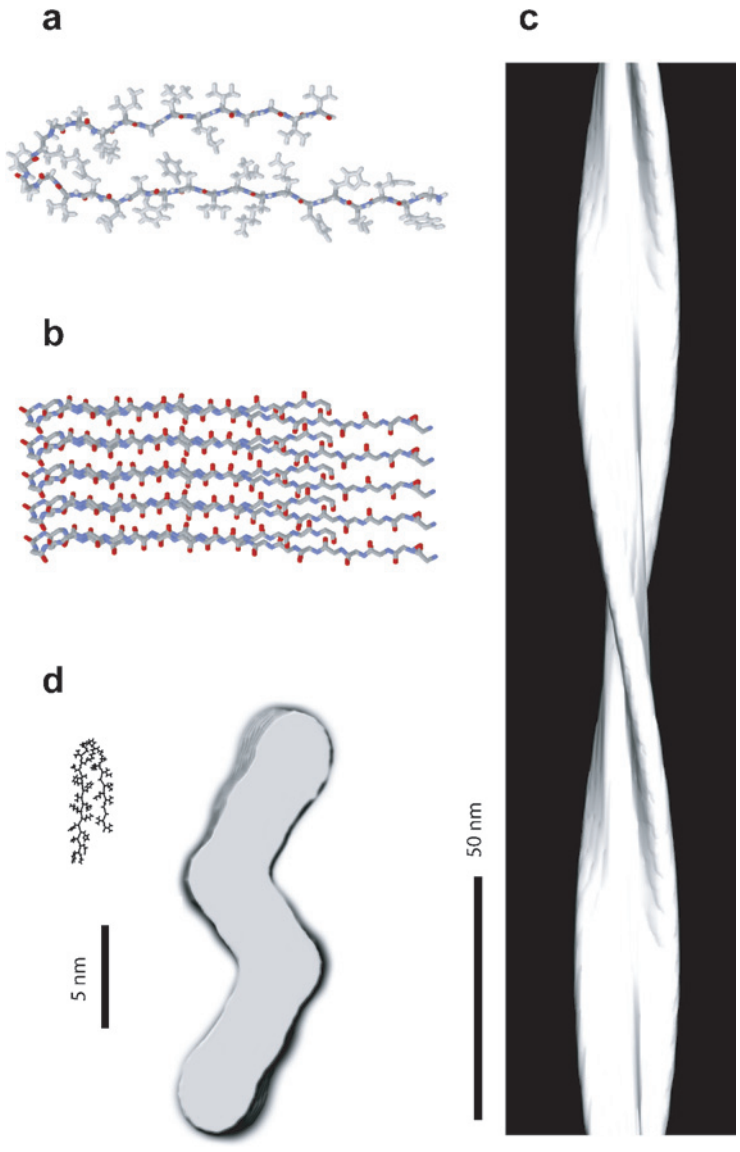

Figure 4. Structure of Aβ(1–40) amyloid fibrils. (*a*) Atomistic model of residues 9–40 of the peptide, after Petkova et al. (2002) [96]. Main axis of the fibril runs orthogonally to the paper plane. The two β-strands interact intramolecularly and in the plane of the paper through their side chains. (*b*) Backbone representation of a stack of five atomistic models of residues 9–40. Main axis of the fibril runs vertically. Backbone carbonyl oxygens and amides are oriented parallel to the main fibril axis so that they allow formation of intermolecular hydrogen bonds with the next upper and next lower peptide arch. (*c*) Side view and (*d*) cross-section of a cryo TEM structure of one Aβ(1–40) fibril morphology at 26 Å resolution [41]. In (*d*) the β-arch structure from (*a*) was scaled to the same size as the density map. The structure of the β-arch was remodelled after original images [96].

Although the resolution of the presented reconstruction is still low (26 Å), it is evident that only some of the previously suggested Aβ peptide conformations are consistent with the cryo TEM structure [41]. A particular good correlation was obtained with a U-shaped Aβ conformation consisting of two juxtaposed β-strands (Fig. 4d).

Why do polypeptide chains form amyloid fibrils?

It has been a central part of our understanding of ordered protein structures that 'the three-dimensional structure of a native protein in its normal physiological milieu (…) is the one in which the Gibb's free energy of the whole system is lowest; that is, that the native conformation is determined by the totality of interatomic interactions and hence by the amino acid sequence, in a given environment' [104]. In other words, a natural polypeptide sequence has only one way to fold up into a native and globular protein structure. The importance of the relationship between amino acid sequence and three-dimensional fold is specifically evident in the context of biological evolution, because it provides the tremendously important link between a linear sequence (and ultimately the genomic information) and a specific three-dimensional state by which a well-defined protein function is determined.

However, amyloid fibrils present a complication to this scenario because they can be derived from naturally occurring and body-own polypeptide chains. But instead of undergoing a native protein folding reaction, polypeptide chains revert into an

alternative state that is effectively the same for all polypeptide sequences, the one of an amyloid fibril. Since the only confirmed common property that distinguishes amyloid diseases from a physiological situation is a conformational and not a sequential property, amyloidoses are considered to be conformational diseases. Of course, examples exist that the conformational transition can be triggered by mutation. But these hereditary forms of amyloidosis are rare, and Alzheimer's disease, for example, occurs mostly in its sporadic form. Mutations promoting amyloidosis often increase the efficiency by which amyloid fibril precursors are generated. Examples hereof are the Swedish mutation or presenilin mutants in Alzheimer's disease [105], mutations that destabilise the native state so that aggregation-competent states become more populated, such as in transthyretin or lysozyme amyloidosis [83, 84]. Thereby, these mutations increase the propensity of the conformational transition towards amyloid fibrils.

The polypeptide chains that form amyloid fibrils *in vivo* and *in vitro* are remarkably diverse with respect to size, sequence, structure and function. Amongst these sequences are full-size proteins and peptides, and the native states of these proteins include cases of different classes of soluble proteins (all alpha, all beta, α/β and $\alpha+\beta$). Furthermore, amyloid formation is not a specific property of only a handful of natural and disease-associated proteins. Inside the test tube, amyloid formation was found to occur also with many other natural or designed polypeptide sequences [31]. Amyloid fibrils can be formed in vitro from myoglobin that is α-helical in its native state [82], several polyamino acids [60] and short designed peptides [106] that are intrinsically unable to fold into globular conformations. Such observations have resulted in proposals that amyloid fibrils represent a generic conformational state of the polypeptide chain [31]. This state is fundamentally different from globular protein states and reflects the intrinsic polymer properties of the invariant polypeptide backbone. Based on this hypothesis, amyloidosis has its molecular basis in natural polypeptide chains that are not able to adopt their native protein conformations and that revert, instead, into the alternative structural state of an amyloid fibril. Understanding the structure of amyloid fibrils, the molecular basis of their formation and the detailed interactions necessary to promote certain structural forms of aggregates *in vitro* and *in vivo* is a prerequisite for developing methods to rationally interfere with these reactions as a basis of new therapeutic approaches.

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