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Minireview

Methods for structural characterization of prefibrillar intermediates and amyloid fibrils

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ABSTRACT

Protein fibrillation is first and foremost a structural phenomenon. Adequate structural investigation of the central conformational individuals of the fibrillation process is however exceedingly difficult. This is due to the nature of the process, which may be described as a dynamically evolving equilibrium between a large number of structural species. These are furthermore of highly diverging sizes and present in very uneven amounts and timeframes. Different structural methods have different strengths and limitations. These, and in particular recent advances within solution analysis of the undisturbed equilibrium using small angle X-ray scattering, are reviewed here.
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1. Introduction

Protein fibrillation is first and foremost a structure related phenomenon. Not only are fibrillar structures exceedingly fascinating formations of an almost esthetically pleasing overall construction. Also, the entire onset of the fibrillation process is defined (perhaps with bacterial functional fibrils as a prominent exception [1]) via refolding of native proteins into temporary conformations of very limited stability, ultimately leading to subsequent formation of suprastructures of high complexity (Fig. 1). The suprastructural formations potentially include several levels of tangling of protofilaments and -fibrils, often resulting in heterogeneous populations of different fibril morphologies [2] (Fig. 2), which may further repack over time. Between the initial destablisation of the native structure and the final tangling of the micrometer long mature structures, lies sequential addition of the individual fibril building blocks, which one by one must add to the growing fibrils. Fibril building blocks may be of mono- or oligomeric nature (e.g. [3,4]), certainly with a markedly modified overall conformation as opposed to the native state, although this overall conformation may be of a less well-defined nature than the typical stable protein structures, which are known, e.g. from the enzyme world. The structural nature of such a building block and structural details of the individual interactions between the millions of protomers of a single protein fibril are important for achieving an understanding of the fibril elongation process and for potential modification, control or inhibition of the process. This has obvious relevance in treatment of amyloid disease, but if adequately controlled, such knowledge may also be of potential relevance in designing functional nanostructures with desired surface properties from biological material. A detailed structural insight into the destablisation of the native fold in the initial stages of the fibrillation process is central for future development of inhibitors of fibrillation, aiming at prophylactic treatment of the associated amyloid diseases, or at stabilisation of industrially produced proteins, e.g. in the pharmaceutical industry. Importantly, several studies indicate that specific oligomeric structures, formed on- or off-pathway during fibrillation and significantly smaller than mature fibrils, may be the primary cause of the cytotoxicity (e.g. [5–7]), associated with several engraving diseases. There is an obvious interest in revealing the structural specificities of such compounds, again to enable rational drug design.

Hence, structural insight is not only key to understanding the fibrillation phenomenon, it is also a prerequisite for the

Abbreviations: 3-D, three dimensional; Aβ, amyloid β; AFM, atomic force microscopy; CD, circular dichroism; EM, electron microscopy; EOM, ensemble optimization method; FTIR, fourier-transform infrared spectroscopy; NMR, nuclear magnetic resonance (spectroscopy); SANS, small-angle neutron scattering; SAS, small-angle scattering; SAXS, small-angle X-ray scattering; ss-NMR, solid-state NMR; STEM, scaning tunneling electron microscopy; ThT, thioflavin T; TTR, transthyretin; WAXS, wide-angle X-ray scattering

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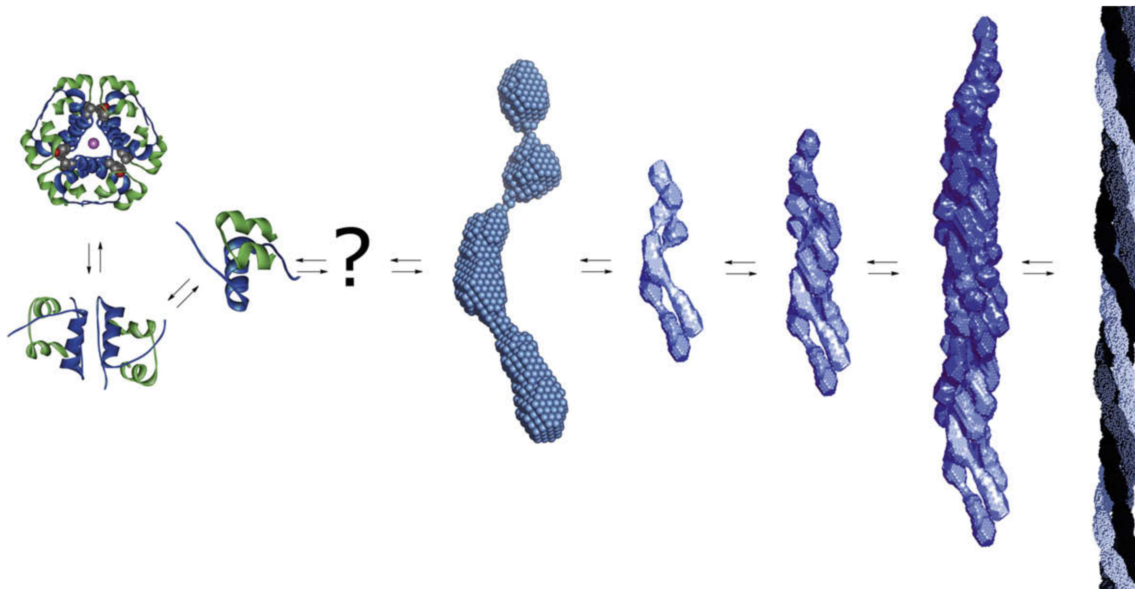

Fig. 1. Illustration of the many equilibria involved in the fibrillation process using insulin as an example. Starting from a native R_6 hexameric form in equilibrium with dimer and monomer states (all built from pdb id 1ev6) a (partially) un-/refolded state is formed. The state is marked by a **?** since no adequate structural information exists at present. Models of an oligomeric state of insulin and the corresponding mature fibrils were obtained by SAXS [4]. The oligomers are presumed building blocks forming the protofilaments, which then intertwine to form the mature fibrils. The mature fibrils shown in this scheme consist of three intertwining protofilaments, but often mixtures of different morphologies co-exist.

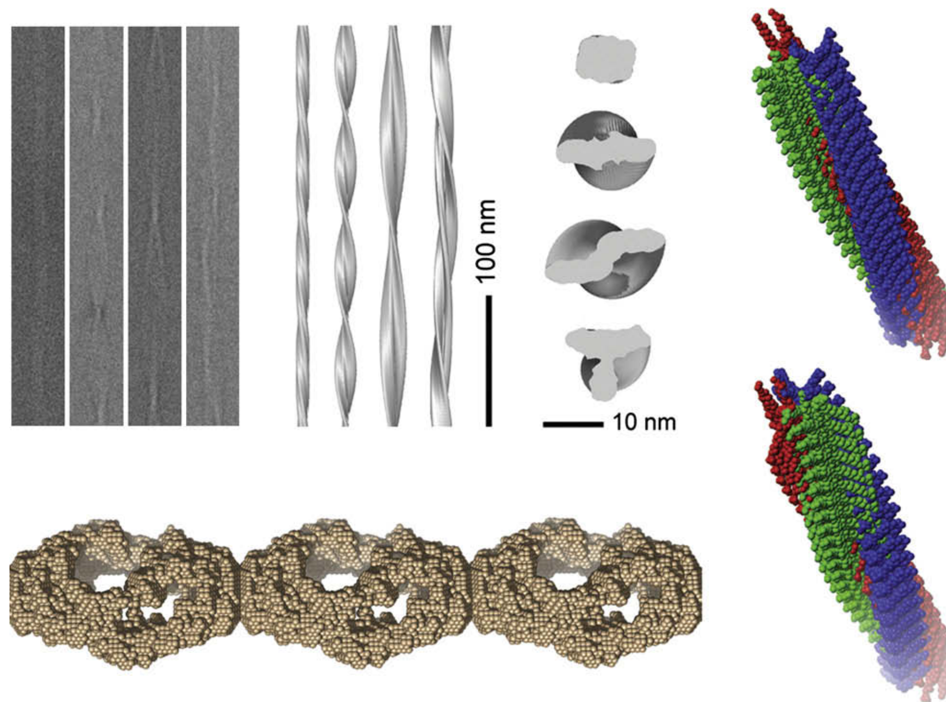

Fig. 2. Examples of models of mature fibrils, obtained from different methods. Top left: Electron micrographs and reconstructions of individual $A\beta_{1-40}$ fibrils. (Selected panels are reprinted from J. Mol. Biol. Meinhardt, J., Sachse, C., Hortschansky, P., Grigorieff, N., and Fandrich, M. "Abeta(1–40) fibril polymorphism implies diverse interaction patterns in amyloid fibrils" pp. 869–877 © 2009 with permission from Elsevier.) Right: Models of glucagon fibrils based on SAXS. The two different structures are formed at different concentrations. The models are built from building blocks of high-resolution native glucagon and fitted to the SAXS-data. (Reprinted from J. Mol. Biol., 387, Oliveira, C.L.P., Behrens, M.A., Pedersen, J.S., Erlacher, K., Otzen, D.E., and Pedersen, J.S., "A SAXS study of glucagon fibrillation", pp. 146–161, © 2009 with permission from Elsevier.) Bottom: SAXS based model of three repeating units of mature insulin fibrils [4]. The model of one repeating unit is an average of several individually calculated ab initio bead models, hence the models are objective.

century-long awaited breakthrough in treatment of amyloid diseases, and also an important factor to meet the increasing demand for protein stabilizers in the growing protein-based industry. All of this said, the associated great basic scientific interest should be emphasized. Fibrillation has been suggested to be a generic property of all proteins [8], yet is only observed in specific cases. An increased comprehension of structural phenomena in fibrillation is inherently related to an improved understanding of protein fold

formation, (de-)stablisation and refolding, all central to our basic knowledge about proteins.

Even so, after a century of constantly increasing focus on fibrillation we do not have an adequate structural understanding of the phenomenon. To a large extent, this is due to the nature of the fibrillation process, most often including a large number of co-existing structural species of greatly diverging sizes – a situation, which complicates almost any type of structural investigation. Add to this the sensitivity of the fibrillating systems to any changes of experimental/external conditions, and the result is an extensively challenging research field, of utmost importance.

Several widespread techniques are being utilized in the study of the fibrillation process and the species involved. Some of these techniques only indirectly add structural information to our understanding of the process. A prominent example concerns the usage of fibril-specific fluorescent probes, which to some extent is the golden standard for detection and characterization of the fibrillation process, and has been used extensively throughout the community for half a decade [9]. However, without atomic resolution information about the molecular interactions between fibrils and probes, we cannot adequately analyse and interpret fluorescence data, let alone rationally design and develop improved probes with altered specificity, e.g. for early oligomers or particular fibrillar morphologies.

This minireview aims at highlighting some of the many recent impressive advances within structural analysis of protein fibrillation and at discussing aspects of the current exciting development within the field.

2. Structural fingerprints – the cross-β pattern

Several definitions of the term 'amyloid' are used. One of the less restrictive, based on biophysical characteristics, is '*a polypeptide, which upon polymerisation exhibits the characteristical cross-β pattern*', as identified by X-ray fiber diffraction studies. Fiber diffraction provides spatial information of frequently occurring distances in the structures. The diffraction patterns (Fig. 3, top right) from (partially) aligned amyloid fibrils display very strong intensities, which in real space correspond to distances of 4.7 Å and approximately 10–11 Å, the former in the meridian plane, the latter in the meridonal plane, hence relating to typical distances perpendicular and parallel to the long axis of the fibrils, respectively [10]. These distances are identical to the typical distances seen in a β-sheet sandwich, hence protein fibrils all seem

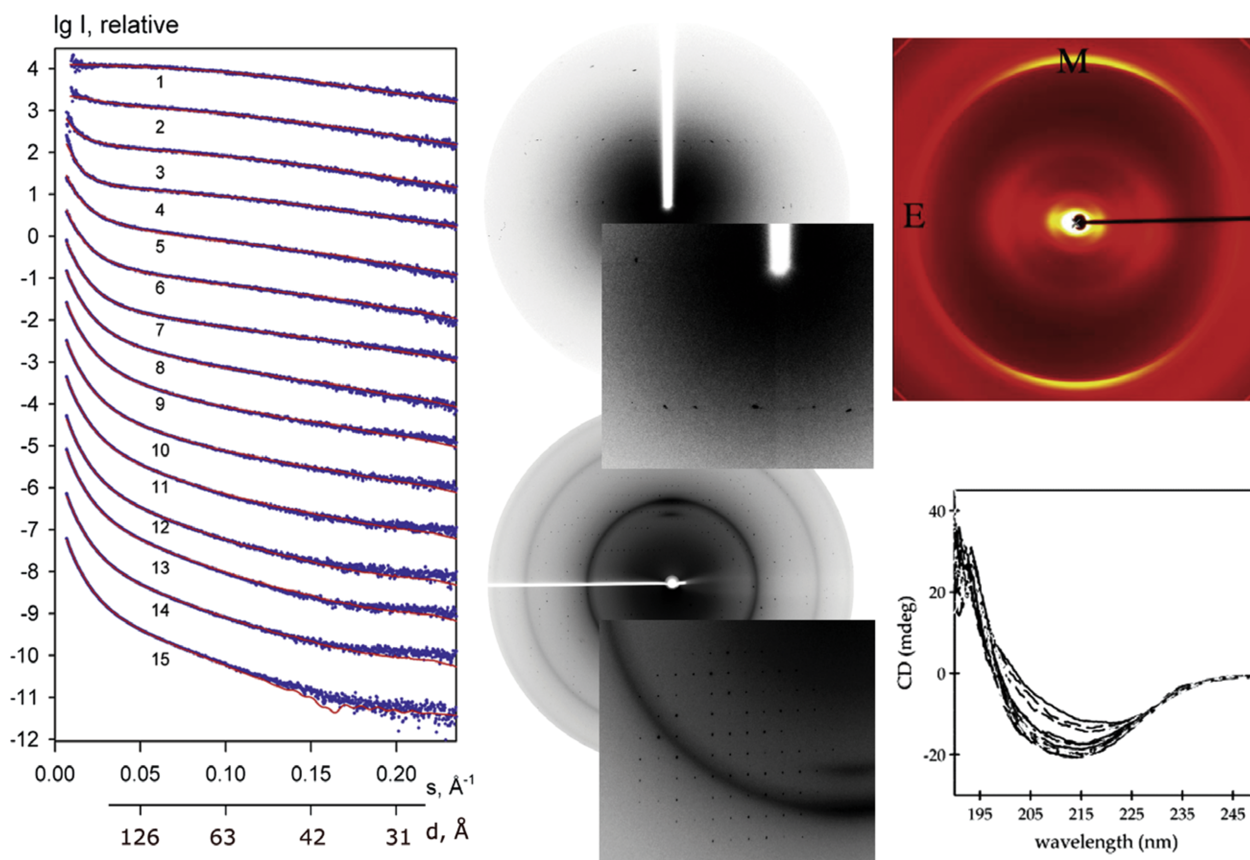

Fig. 3. Examples of raw data types, from methods applied in the study of amyloid structure. Left: Background subtracted radially averaged SAXS data curves from the kinetic study of insulin fibrillation [4]. From top to bottom, data is collected at increasing time points throughout fibrillation and the scattering intensity I is plotted as a function of $s = 4\pi\sin\theta/\lambda$; 2θ is the scattering angle and λ is the X-ray wavelength. Each data curve is translated to ease visualization. The nominal resolution is indicated as $d = 2\pi/s$. In this particular case fitting was obtained to approximately 20 Å resolution, but resolution in SAXS data often extends to medium resolution (sub-nanometer). Middle top: Crystal diffraction pattern from the GNNQQNY peptides (courtesy of A.Ø. Madsen and D. Eisenberg). Middle bottom: crystal diffraction pattern from insulin at fibrillation-promoting conditions (pH 1.6) (V. Fodera and B. Vestergaard, unpublished data). Insets show how the spacing between reflections differ depending on the size of the unit cells of the crystals, hence peptides (top) pack in very small unit cells and insulin (bottom) packs in a relatively larger unit cell. In the diffraction image from insulin crystals, diffuse scattering from ice on the surface of crystals is evident. The diffraction extends to 1.8 Å (GNNQQNY peptides) and approximately 1.9 Å (insulin), respectively. Right, top: Fiber diffraction data from partially aligned insulin fibrils (adapted from [4]). The most dominant reflection is at 4.8 Å at the meridian (M), and weaker signals at 10.5, 14.5 and 30 Å are visible on the equator (E). Right, bottom: time-evolution of far-UV CD signals from fibrillating concanavalin A. (Reprinted from Biophys. Chem., 125, Vetri, V. Canale, C., Relini, A., Librizzi, F., Militello, V., Gliozzi, A., and Leone, M., "Amyloid fibrils formation and amorphous aggregation in concanavalin A", pp. 184–190, © 2006 with permission from Elsevier.)

to share a common structural motif, namely a spine formed by extensive β-sheets with individual β-strands perpendicular to the fibril axis (Fig. 4). Existence of large proportions of β-strands in fibrillated protein is easily confirmed, e.g. using Fourier-transform infrared spectroscopy (FTIR) or circular dichroism (CD). This feature seems to be the main structural feature of all amyloid fibrils, irrespective of the native protein fold of individual proteins. Hence, significant refolding of the proteins is a prerequisite for fibrillation in most cases.

Quasi high-resolution features have been modelled when interpreting fiber diffraction data, often in combination with complementary high-resolution structural information. Recent examples include the analysis of short peptide-based fibers [11,12] or longer protein fragments [13] in a powerful combination with solid-state NMR (ss-NMR) (see later section). This concept would become even more promising by including structural information from additional complementary methods, e.g. from small angle X-ray scattering (SAXS) and X-ray crystallography. This would further diminish the inherent uncertainties when modelling structure based on underdetermined data.

3. Specifications of individual structural states

The cross-β fibrils are the end product of the amyloid fibrillation process. The fibrils are micrometer long, predominantly unbranched aggregates, often consisting of several intertwining protofibrils. The formation mechanism is heavily debated (e.g. [14–16]) but all agree that several structural states appear during the process. Fig. 1 shows an example of a number of equilibria, involved with insulin fibrillation. One may consider the starting point of fibrillation as being divided into two distinct structural classes: those where the monomeric native protein adopts a globular fold (as monomers or in some cases, such as insulin, predominantly as native oligomers that must first be disrupted), and those where the protein is natively unfolded (classical examples being α-synuclein, the amyloid β (Aβ) peptide and amylin). In both cases the monomeric protein partly refolds into a non-native structure of low thermodynamic stability. At present, no high-resolution structural information exists from a significantly refolded monomeric fibrillation precursor, although several destablised structures are described. From this structural equilibrium, an important component for the understanding of fibrillation – the nucleus – may form. The nucleus is per definition the thermodynamically least favorable species on the fibrillation pathway. It is believed to consist of a critical number of associated un/refolded protomers, and upon formation of the nucleus the elongation phase of fibrillation initiates, during which either non-native monomers or oligomers associate to form the protofibrils. The interest in the nucleus structure derives firstly from the basic assumption that the nucleus is the smallest possible unit that defines the repeating structure of the whole fibril. Hence, the structural features of this particular species are the exact structural features that define the entire fibril structure, and if the nucleus is adequately structurally described, so is the fibril. But the large interest in the nucleus also relates to a number of studies, which indicate that oligomeric prefibrillar structures, and not the mature fibrils, are the cause of cytotoxicity (e.g. [6]). Whether such toxic oligomeric structures are on- or off-pathway is not clear, hence the toxic species may or may not be identical to the nucleus. The final structural assembly can include longitudinal intertwining or lateral assembly of individual elongated protofibrils, with varying levels of complexity. Often, several fibril morphologies co-exist in solution [17–19]. The molecular weights of individual species cover a range from Da (peptides) to MDa (repeating units of mature fibrils), and most species co-exist in equilibrium. Truly a challenging system for analysis.

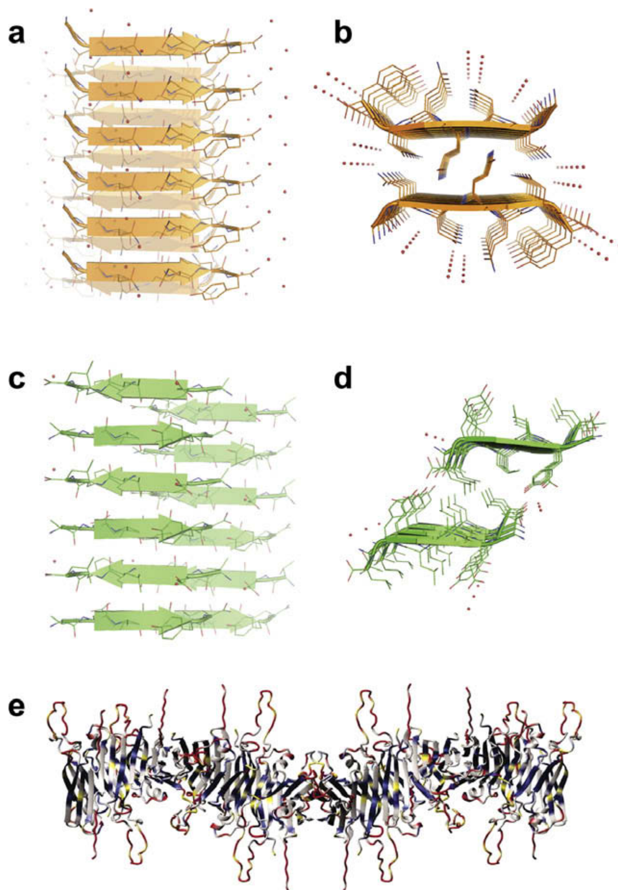

Fig. 4. Examples of results from high-resolution methods. Crystal structures of peptides GNNQQNY (a, b) and VEALYL (c, d). A few strands of the dry-zipper are illustrated in side and top view. The β-sheets are either parallel (GNNQQNY) or anti-parallel (VEALYL). The peptide figures were produced using PyMol [86] based on deposited structures (pdb id 1yjp [49] and 2omq [50], respectively). A model of TTR fibrils (e) (reprinted from [63]) based on the crystal structure of the native protein and solution NMR experiments on fibrillated TTR is shown. Colors indicate relative protection factors obtained through H/D exchange experiments.

4. Direct visualization of fibrils and oligomers

Electron microscopy (EM) is traditionally considered a low-resolution method, but has the huge advantage of being a direct method, hence avoiding the phase-problem inherent to scattering and diffraction techniques. Both transmission and scanning EM has been used extensively for low-resolution characterization and visualization of mature protein fibrils. For biological specimens cryo-EM greatly improves resolution, and impressive results have been obtained [2,17]. Reconstructions reveal the level of branching, helical pitch of individual fibrils, number and overall dimensions of protofibrils/-filaments, and more recently with the improved resolution even the first traces of the main-chain are now appearing [20]. The latter achievement has been made possible by recent advances in reconstruction methods and hardware. Three dimensional (3-D) reconstruction may greatly benefit from averaging over the symmetric particles, and can be performed from individual subpopulations within cryo-graphs [17,20], based on either subjective or objective (software based) selection [21]. In fact, since individual fibrils have a high number of structural repeats, it is possible to perform averaging over a single fibril. This has been used to reconstruct as many as 12 different fibril morphologies from one individual sample of Aβ(1–40) [22], (Fig. 2, top left),

hence proving that the same protein can form different mature morphologies, perhaps guided by local variations in protein concentration or other slight deviations. Even higher-resolution imaging may be obtained but assumingly requires significantly increased control over the fibrillation process in order to generate more homogeneous samples, which still is preferable over the selection processes after data collection or, which alternatively supports the effort. The method is thus ideal for the visualization of mature fibrils with great promise for higher-resolution reconstructions in the near future. Electron microscopy has also been used to visualize isolated apparent off-pathway protofibrillar structures, e.g. the annular cold-induced α-synuclein oligomers, particularly visible among familial mutant A53T preparations (Fig. 5, right), or corresponding mixtures of tubular, annular and spherical structures in wild-type and familial mutant A30P preparations [23].

In contrast to the averaging methodologies often used in cryo-EM reconstructions, scanning tunneling electron microscopy (STEM) and atomic force microscopy (AFM) produce single particle 2D profiles. The theoretical resolution is extendable to sub-nanometer, i.e. approaching high-resolution and indeed rather detailed information has been obtained (Fig. 5, left). Smaller particles are easily described, and remarkable results have been obtained characterizing on- or off-pathway oligomers in various systems (e.g. [24,25]). One impressive example is the Aβ-peptide oligomers formed by apparent end-to-end associations, where what appears to be individual β-strands are vaguely visible in protomers [24]. Such β-strand rich appearance would be expected for an on-pathway oligomer, it is however not conclusive if the oligomers are fibril building blocks or not. Fully assembled fibrils of elastin-derived polypeptides have been characterized by AFM/STEM, revealing the overall, albeit lower-resolution features. Importantly, both of the latter studies were performed without metalcoating of the biological samples, which, apart from being highly desirable in eliminating the risk of modifying the structures by the coating process, also shows that at least the analysed fibrils have interesting charge-transfer properties. AFM may be used quantitatively (e.g. [26,27]) or qualitatively. Qualitative AFM is often used in a semi-time-resolved manner, examining the distribution of species during the aggregation progress, e.g. compared under different solution conditions [28]. It may also be used in closed fluid cells, which enables in situ analysis of the structural effect of, e.g. small-molecule compounds such as the example describing disaggregation of Aβ-peptide oligomers [29] by small-molecules with anti-cytotoxic effects in vivo. Hence, AFM is an important method for direct time-resolved detection, e.g. of oligomer formation. Of perhaps particular interest is the option to use biologically relevant wetted surfaces, exemplified by the primarily AFM-based study of

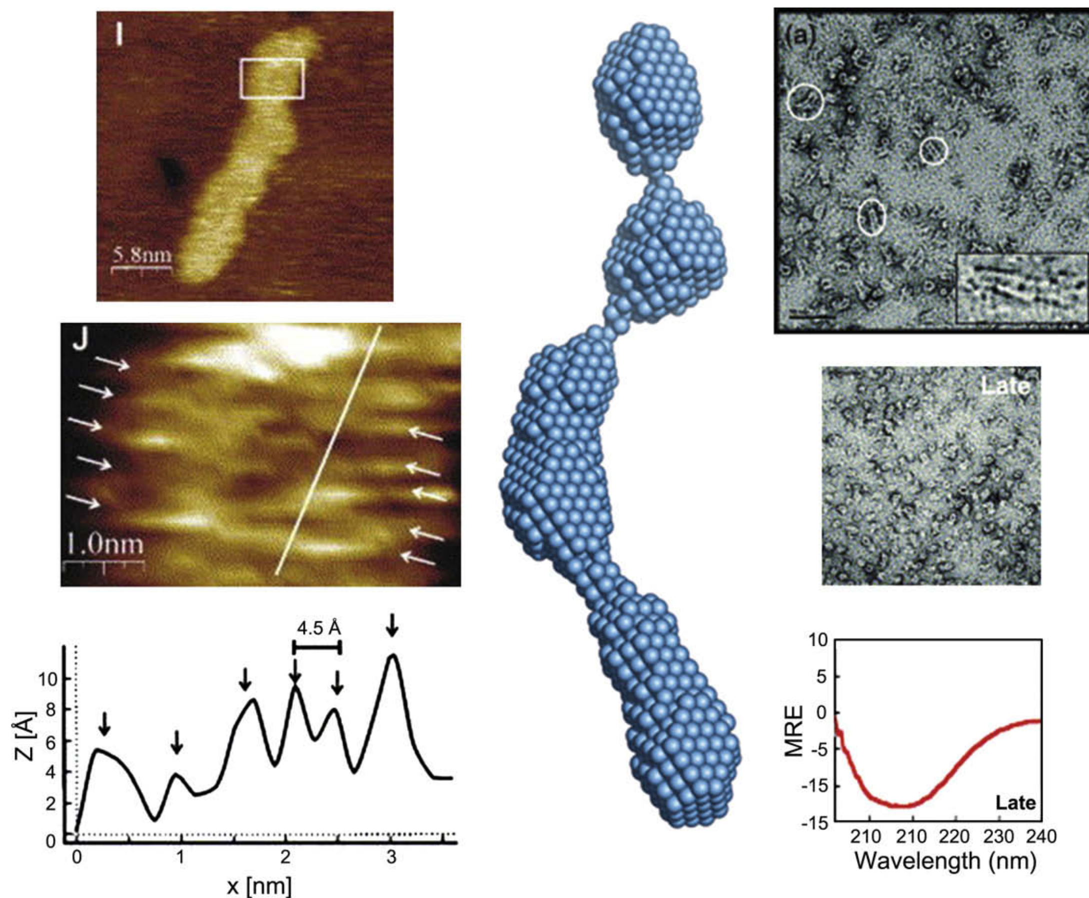

Fig. 5. Examples of oligomeric structures obtained by different methods. Left: Scanning tunneling microscopy of a single $A\beta_{1-40}$ oligomer, magnification corresponding to the white box in the top panel and the surface topography along the white line. (Reprinted from J. Struct. Biol., 155, Losic, D., Martin, L. L., Mechler, A., Aguilar, M. I., and Small, D. H., "High resolution scanning tunnelling microscopy of the β-amyloid protein (Aβ1–40) of Alzheimer's disease suggests a novel mechanism of oligomer assembly", pp. 104–110, © 2006 with permission from Elsevier.) Middle: SAXS-based solution structure of the insulin on-pathway structural nucleus. Reprinted from [4]. The model is obtained from decomposition of the data measured from the undisturbed equilibrium of the evolving fibrillation. Right: Electron micrographs of A53T α-synuclein oligomers and far-UV CD spectrum showing the high β-sheet content in the late fraction of gel-filtrated oligomers. (Reprinted from J. Mol. Biol., *322*, Lashuel, H.A., Petre, B.M., Wall, J., Simon, M., Nowak, R.J., Walz, T., and Lansbury Jr., P.T. "α-synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils", pp. 1089–1102, © 2002 with permission from Elsevier.)

amylin deposits and aggregation on planar lipid surfaces of varying composition [30]. Surfaces play a significant role on amyloid aggregation propensity and kinetics [30–33], and on the resulting morphologies of fibrils, and all the above-mentioned techniques suffer from extensive surface contact between specimen and sample support. Although the same applies to total internal reflection fluorescence microscopy, this method is highly interesting since it allows a time-resolved direct visualization of fibril growth and branching [18,34].

5. Small angle scattering in solution

One major advantage when applying solution small angle neutron or X-ray scattering (SANS/SAXS) to the study of amyloid formation is exactly the lack of surface effects. Small angle scattering (SAS) techniques are low-/medium resolution well-established methods for the structural investigation of virtually any type of particle on the nano-scale (resolves particles in the range 1–100 nm). Relatively recent significant improvements of data analysis methods when applying SAXS/SANS to biological solutions [35] have revitalized the field, in particular by the development of the elegant and appealing *ab initio* modelling methods [36]. In combination with more traditional analysis such as the derivation of overall dimensions, pair distance distribution functions [37] and fitting to known high-resolution structures [38] potentially applying rigid-body modelling [39] provides structural descriptions of biomacromolecules in solution quite complementary to other low-/medium-resolution methods (e.g. cryo-EM) and high-resolution methods (e.g. X-ray crystallography). There are virtually no limitations on the experimental conditions applied and this is crucial in enabling studies of the amyloid process, since particular conditions are necessary to promote fibrillation. Of utmost importance, SAS data are additive, meaning that it is possible to separate signals from one particular species in solution from a collected signal, which may be exploited for kinetic studies of fibrillation. Accordingly, we used ab initio modelling based on the SAXS data (Fig. 3, left) to describe the low-resolution three-dimensional structure in solution of a co-occurring oligomeric structural nucleus (Fig. 5, middle panel) and the repeating fibril unit of insulin [4] (Fig. 2, bottom). We revealed that an oligomeric helical species consisting of approximately six protomers accumulated in solution, and that its concentration directed the rate of fibril growth. In accordance, we proposed that this structural nucleus was the building block of insulin fibrils under the conditions examined, and could suggest a mechanism of aggregation, which would result in hollow tubular protofilaments of a size and molecular weight in accordance with the dimensions of the mature fibrils (Fig. 1) [4]. This would also imply that the structural nucleus would be in a primarily β-sheet conformation, which again suggested that almost the full length of the insulin backbone is refolded into β-strands, in accordance with e.g. CD and FTIR observations [40]. Our results were subsequently confirmed by neutron scattering experiments [41] and this work further debates the aggregation mechanism involving these same structural nuclei. Our current studies of both insulin fibrillation under different experimental conditions and other protein systems (Groenning M. et al.; Giehm, L. et al., unpublished work) elaborate on the initial findings, and further add to an understanding of fibril formation mechanisms but also reveal distinct differences in the overall conformation of different on-pathway oligomeric species. A recent study of glucagon fibrillation by Oliveira et al. [42] very elegantly extends the method by using a completely model free approach to isolate scattering fractions from the on-pathway intermediate species. Also in this study, an oligomeric species is present in relatively large quantity. The oligomer has an elongated shape, and the overall dimensions were comparable to the insulin nucleus albeit differing at more detailed level [42]. This is striking when taking into account that the data are from two unrelated protein systems with different native sequence, fold and oligomeric distribution. The mature fibrils were here described by fitting rods, composed of a high number of high-resolution native building-blocks, to the SAXS data (Fig. 2 and [42]). On a related topic, spider silk fibroin aggregation has been studied by a combination of SAXS, wide angle X-ray scattering (WAXS) and Raman spectroscopy [43]. Here, a microfluidic flow cell where protein is mixed into a fluid of lower pH stimulating silk formation has been used. X-ray exposure at increasing distance from the fluid-cell mixing point corresponds to increasing timepoints after initiation of fiber formation. Albeit per definition not being an amyloid, the silk has a number of features quite similar to that observed for amyloids (such as extensive β-sheet formation perpendicular to the long axis of the fiber and an overall large-scale aggregation via refolding), and also an elongated pre-fiber structure of fibroin is observed [43]. The study is mentioned here, in part for the resemblance of the results to the previously mentioned studies, in part for the microfluidic approach, which may be of future use for studies of amyloid formation also, although the foreseeable problems with clogging of the microfluidic channels need to be resolved. It is nevertheless of importance to think in creative ways of preparing samples for structural studies of amyloid.

Several other studies report the use of SAXS or SANS for studying various amyloid-related structural features. Most of them do not go as far as actual ab initio modelling, but important conclusions can still be reached based on the biophysical parameters derived from the data. A few examples should be mentioned here. One is the analysis of the early stages of α-synuclein fibrillation, where deconvolution of data based on two-state kinetics suggests the formation of a prefibrillar heptamer [44], which in contrast to the native unfolded state has a more globular appearance. This study is combined with NMR-analysis, which pinpoints residues seemingly involved in contacts during fibrillation. In two other studies, hen egg white lysozyme fibrillation is studied by SAXS and SANS [45,46], examining the effect of different experimental conditions on the resulting fibrillation state. Finally, an interesting method, considering contributions to the data from polydisperse systems, is the recent solution analysis of native Tau protein [47], using the program Ensemble Optimization Method (EOM) [48].

If applying SAS to analysis of the amyloid process, it is important to carefully consider both the experimental conditions and the data collection strategy. Although solution scattering data from mixtures can be deconvoluted into the individual scattering curves, this obviously only applies if it is possible to assign the accurate number of individual states, and if each state is adequately represented in solution. Also, data from homogeneous solutions of one or more of the individual states must be obtained or reliable high-resolution structures as representative particles must be available. Hence, starting conditions should always be very accurately analysed, and it is very important that conditions evoke a relatively homogeneous final mature fibril state also. The alternative is an analysis performed on data from averages of several states, also after attempting deconvolution. This will in the best case be meaningless, and in the worst case be directly misleading. This warning specified, solution scattering offers a unique mean of analysis of the amyloid process. The opportunity to obtain direct structural data while experimental conditions can be fine tuned to the individual system (in contrast to being defined by methodological limitations), that surface effects are negligible, and – in particular – that the structural evolving equilibrium is left undisturbed during measurements, is not offered by any other structural method.

6. High-resolution methods

From the X-ray diffraction pattern of a single crystal (Fig. 3, middle) the electron density, and thus the coordinates of all the atoms can be determined, thereby attaining atomic resolution structural information from the sample. The ultimate requirement in X-ray crystallography is diffracting crystals, which in general only forms from highly homogeneous solutions, which is not the case for fibrillating solutions. The resulting structural information describing near-monolithic 'snapshots' of protein structure will perhaps never adequately describe fibrillar species, which in solution exist in dynamically changing equilibria between several structural states. However, the high-resolution information that has been obtained from amyloid-relevant systems is of immense importance to our slowly improving understanding of the structural features underlying fibrillation.

A large number of peptide fragments from fibrillating proteins have been studied theoretically to predict their fibrillation propensity. Subsequent experimental studies revealed numerous fragments (typically 6–7 amino acids long) forming fibrils with the characteristic cross-β pattern. In addition some of these peptides form nano- and microcrystals [49–51], which along with the impressive development of high-quality micro/nano-focus beamlines [52,53] has enabled the structure determination of several amyloidogenic peptides originating from a range of fibrillating proteins [49–51]. These structures reveal a high level of self-complementarity and also demonstrate a very tight packing of neighbouring β-sheets in the so-called *steric zipper* (Fig. 4). The β-sheets in these structures are straight, due to the crystal packing, thus an open question is still how this relates to the twists and intertwining of protofilaments and mature fibrils as observed by AFM, STEM, etc. Even for such model peptides the comparison of the structures in nano-crystals and the modelling of quasi-atomic resolution structures onto fibrils is not straight-forward [54]. Finally, the position of the remaining polypeptide chains in the corresponding full-length protein is impossible to predict, or even imagine, based on the peptide structures. These problems, however, by no means reflect on a lack of importance or quality of the high-resolution structures, on the contrary. The mentioned problems only highlight the lack of information, which still dominates the field, and emphasizes the importance of struggling for further advances of the same standard as these impressive achievements. However difficult to incorporate into full-length models or mature fibril structures, the high resolution crystal structures do contribute to the understanding of the underlying specific structural features and how differences in these features may result in mature fibrils with different morphologies. In the peptide structures motifs with parallel in-register arrangement of the β-strands appear to dominate [50,55].

The peptide structures have been used for modelling onto lower-resolution structures [11,12]. Some ambiguity obviously remains in such approaches, but these studies are perhaps examples of a useful future approach. It seems highly relevant to combine several structural methods with different resolution and experimental restrictions, such as to bridge from high-resolution structures, e.g. of isolated fragments, to low-resolutions structures of mature fibrils or other on- and off-pathway species. As an example, the insulin structural nucleus described based on SAXS-data [4] has an overall structure and dimensions that indicate that most, if not all, of the protein must form β-strands (see [4] for details). At present, it is not possible to map the high-resolution structures of insulin-fragments [49] onto the structural nucleus in any meaningful way, on the contrary, the two studies to a certain extent are contradictory. However, if a structural nucleus is formed in solutions of fibrillating peptides, and if this structural nucleus indeed is on-pathway, then the conformation of individual peptides within the nucleus should be related to the crystal structure conformations.

Structural information of the fibril state is available through ss-NMR as described very well by Heise [56]. In ss-NMR the ^{1}H signals are not observable, thus ^{15}N and ^{13}C labeling is necessary, which can be difficult to obtain for larger recombinant proteins, but the labeling has advantages. Dividing the labeling can be utilized in the segmented assignment strategy [57] and labeling can also be exploited to distinguishing between parallel and anti-parallel arrangement of the β-strands through single site labeling [58,59]. Distances between single sites of 4.7–5 Å can only be fulfilled in the parallel in-register β-strand arrangement, whereas longer distances must be combined with additional constraints to separate off-register and anti-parallel arrangements. Thus the method can also uncover the (approximate) registry of the β-strands, and along with distance restrains elucidate the molecular arrangements and motifs at a fairly detailed level. Polymorphism of fibrils is believed to be caused by underlying differences of the packing at molecular level, and thus the different forms will be distinguishable by NMR as shown for Aβ by Petkova and co-workers [60].

ss-NMR studies have also enabled direct comparison of peptide nano-crystals and fibrils [54]. For one of the published peptide zipper crystal structures (sequence GNNQQNY) three co-existing fibrillar forms were found (or three structural conformations co-existing in one fibril) along with the two known crystal forms [49,50]; detailed comparison of individual chemical shifts and Tyr-Tyr interactions reveal several differences, thus also exemplifying the complexity of correlating crystal structures and fibrils even for the model peptides. Valuable information on the fibrillar core region of full-length proteins as obtained for α-synuclein [59] is an excellent example of the type of information required in the complex puzzle of correlating peptide fragment high-resolution structures to the low-resolution information from the corresponding full-length proteins. Solution NMR has provided high-resolution structures of partially re-folded monomers, which however are clearly not in a significantly refolded β-sheet rich conformation directly relevant for fibril formation [61]. Solution NMR can also complement ss-NMR with information on solvent accessibility through H/D exchange experiments revealing buried hydrogen bond interactions. A very fine example of the results of a joint solution and ss-NMR study is the Aβ structure [62]. For transthyretin (TTR) the solvent accessibility of the fibrils were compared with the native TTR crystal structure resulting in a proposed model of the TTR fibrils with TTR retaining a native-like structure in the fibrils [63] (Fig. 4, bottom). Ss-NMR may also successfully be combined with other methods, e.g. with fiber diffraction, TEM and AFM as in the case of the de novo designed ccβ peptide [64] or with mutational studies as for a domain of a human transcriptional activator [65].

X-ray crystallography or solution NMR can also be employed to obtain relevant information of native and native-like structures, e.g. the complex of prion protein and RNA [66], a domain-swapped Rnase A [67], Tau protein [68] and acylphosphatase from *Sulfolobus solfataricus* [69].

In conclusion, the high-resolution methods all provide extremely important information on the details of the fibril structure. These methods will remain of utmost importance in the amyloid field, in spite of the significant associated challenges. In particular, high-resolution methods are crucial for obtaining the atomic coordinates revealing not only the structures themselves, but also potentially their interactions with specific amyloid probes (such as the fluorophores thioflavin T (ThT) or congo red) or potential small-molecule or peptide based inhibitors of fibrillation.

7. Indirect complementary methods

Several techniques offer complementary indirect structural information. Structural models based on any of the mentioned techniques have limitations, thus for increased validation and application it is important to use additional information. This can be done by combining low- and high-resolution methods, and/or by correlating with indirect complementary methods. Here we will mention a few of the most relevant complementary methodologies in the field of amyloid structure, but a full list of the many techniques applied is beyond the scope of this minireview. Changes in secondary structure content can be followed through CD during the fibrillation process (Fig. 3). An example is the study of the kinetics of Concanavalin A by Vetri et al. [70] where AFM and ThT fluorescence measurements are also employed. Several variations of Raman spectroscopy and microscopy are also applied in the study of amyloids. Multiple examples of FT-IR approaches used in the analysis of amyloids are described in the review by Hiramatsu and Kitagawa [71]. The study of amyloids also benefits from advances within the field such as the use of drop-coating-deposition-Raman [72]. In addition RAMAN microscopy is used in *post mortem* studies of amyloid deposits formed in the brain of human and, e.g. for in vitro characterization of α-synuclein fibrillation [73] (and references herein).

Other interesting methods providing information on the fibrillation process and species are, e.g. neutron reflectometry in which important molecular interactions are elucidated by studying interactions of the fibrillating proteins with various charged lipid membranes [74] and ultrasound velocimetry, densimetry and calorimetry along with FTIR was used in a study of insulin fibrillation [75].

8. Modelling

The application of theoretical calculations and modelling to answer questions relating to fibrils and the fibrillation process spans widely from all atom molecular dynamic simulations studying the specific interactions involved between peptides, over larger scale simulations of the fibril formation to the prediction of fibrillation propensity from a given amino acid sequence.

Many different approaches have been employed to obtain the latter information. Several of these methods rely on structural information either from proteins in general [76] or have been based on specific structural motifs (e.g. parallel β-strands) (Fig. 4) [77–79]. The selection of sequences for the peptide crystal structures is an example of the success of this approach.

Some fundamental features of fibrillation make the application of existing modelling approaches quite complex: the fibrils are in a local minimum in the energy landscape, whereas the nucleus per definition is the thermodynamically least favorable species in the process. This complicates the theoretical work on describing the early stages of the fibrillation process, as these features per definition are up-hill in energy terms. Another problem complicates theoretical calculations: Most methods use experimental high-resolution structures as a starting point of simulations. There is however an, enormous gap in knowledge between structures of the native state of several fibrillation-prone proteins and the above-mentioned structures of isolated peptides in an amyloid-relevant conformation.

Simulations and predictions are still very important tools in identifying, e.g. significant energy contributions, intermolecular interactions as well as explaining (or predicting) oligomer sizes.

All atom simulations are the most accurate calculations, but also the most CPU demanding and thus only relatively short time frames (typically nanosecond scale) are simulated. These types of calculations require a starting molecular geometry and therefore benefit from using crystal or NMR structures as starting points. The method can pinpoint important native interactions and evaluate the energy involved in a given refolding process marking the starting point of fibril formation. A simulated disaggregation of the GNNQQNY zipper [80] is an example of the use of crystal structures, studying the stability of the structure in a reverse manner. Larger scale simulations using simplified peptide models ("intermediate resolution") have been employed to study aggregation of a finite number of model peptides [81], which evaluated different fibril growth mechanisms.

An interesting example of all-atom modelling is the ccβ peptide (17 residue de novo synthesized peptide, [82]) where the free energy landscape was surveyed to find several local minima for structures containing three β-strands. Hence, even for this relatively small peptide several pathways from the native-like coiled-coiled structure could be found, highlighting the complexity of these systems. The latter study elegantly demonstrates the fundamental problems associated with modelling without experimental data, and at the same time holds promise for the application of advanced modelling, once relevant high-quality data of individual states, or a limited number of states, is available.

Even modelling a limited number of states, described via high-quality experimental data, is troublesome. Perhaps one particular class of development is of the greatest need within modelling of fibrillation, namely ensemble modelling. Fibrillation is in essence, at any timepoint, an evolving equilibrium of different structures, and in order to describe intermediate states adequately, it is important to consider ensembles of structures. It is important to objectively estimate the correct number of structures in such an ensemble, when fitting to data. Too few structures will result in underfitting (relevant conformations are missing even if the result fits to the data), while too many structures will result in overfitting (one is essentially modelling noise, which will improve the fit, but not include meaningful structures).

In SAXS, the data can be deconvoluted using singular valued decomposition or simply by applying the lowest possible number which will adequately describe the data. This was successfully applied in the previously mentioned amyloid studies [4,41,42]. Another approach is to select a given number of structures from a pool of plausible structures, and fit a linear combination of such structures to the data curves, e.g. as is done in the program EOM, developed for SAXS and NMR [48]. Neither singular value decomposition nor EOM addresses the problem of selecting the optimal number of structures in a statistically fully adequate way.

As a final mentioning: the large-scale conformational changes involved with initial refolding limits the usefulness of molecular dynamics simulations, partly due to computational limitations. Rather, future improved ab initio protein structure prediction methods may prove to be adequate for modelling of the refolded amyloid state.

9. Outlook

Above, many of the limitations and challenges by applying different structural methods have been mentioned. Various approaches may be applied in order to overcome the difficulties. A few suggestions should be summarized here. Firstly, the combination of several structural methods is appropriate, in order to compensate for the shortcomings of one method by the inclusion of another. Importantly, SAXS/SANS based ab initio models from time-resolved analysis must be included when combining high- and low-resolution methods. These structures are highly complementary in nature to those obtained from crystallography,

ss-NMR, fiber diffraction or various microscopy methods, and may be a linkage between several other methods.

The emerging technique of time-resolved wide angle X-ray scattering [83] could potentially be of interest also for fibrillation studies, although several technical problems need to be solved first. Such data are a prime example of the need for an improved modelling methodology, including true ab initio methods on ensembles of proteins, a need already significant when dealing with non-amyloid systems [83] and assumingly even more pronounced with the large structural conversions expected in fibrillation.

A particular challenge in the field is in vivo characterization. Comparative studies of in vitro and in vivo generated fibrils is one step, but with time more radical approaches are needed such as, e.g. the NMR structure determination recently demonstrated from living cells [84] or the challenging and promising tomography, e.g. reviewed by Nordberg [85]. However manifold the challenges, they are significantly outnumbered by the needs and interests in obtaining further structural understanding. There is significant interest within research fields spanning from basic science to drug development and materials design, hence there is a natural drive within the community towards obtaining future results even more impressive than what has already been pointed out here.

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