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Review Article

DIAGNOSTIC TOOLS FOR STRUCTURAL CHARACTERIZATION AND ELUCIDATION OF FIBRILS AND THEIR PRECURSORS IN AMYLOID FIBRIL FORMATION PATHWAY

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Abstract: The amyloid fibrils and their precursors are supposed to be responsible for several neurodegenerative diseases. Advances in recent experimental techniques rationalized our understanding on the mechanism of the amyloid fibril formation. The goal of this review is to revisit the various techniques used to diagnose the structural features of amyloid fibrils and their precursors, for a comprehensive view of the available tools, their advantages and disadvantages. The review will serve as a stepping stone for detailed understanding of each technique and its use as per specific requirements of a biological problem.

Keywords: Amyloid fibrils; oligomers; pre-fibrils; intermediate structure.

1. Introduction

It is now well accepted that amyloid fibril formation is the hallmark of many diseases including fatal neurodegenerative diseases such as Alzheimer's, Parkinson's disease etc. (Chiti and Dobson, 2006). Apart from this, fibril formation has been shown to be a generic stable structural state of proteins along with native and unfolded state (Chiti *et al.*, 1999; Rochet and Lansbury, 2000; Uversky and Fink, 2004). For understanding of the properties and effects of amyloid fibrils, the determination of molecular structure of the fibrils is a must. A variety of precursors such as intermediates obtained from folding/unfolding of native proteins, oligomers, protofibrils exist in fibril formation pathway (Scheme 1). Recent studies have shown that quite often the precursors are more toxic than fibrils themselves (Bucciantini *et al.*, 2002; Janson *et al.*, 1999; Klein *et al.*, 2001; Uversky and Fink, 2004). Thus it is not only important to study structure of fibril only but the understanding of structural features of precursors is needed as well. The knowledge of structure of precursors also helps in elucidating the mechanism which is essential for developing effective therapeutics against these devastating diseases. Therefore elucidation of structure of fibrils and their precursors have become central issue among various fields of protein research. This is not an easy task since it requires the complementary data from various techniques and careful analysis. Several reviews are available which discuss about techniques, however, their main focus was either on the structure elucidation of fibrils (Langkilde and Vestergaard, 2009; Li *et al.*, 2009; Saha and Deep, In Press) or structure elucidation by a particular technique (Hoffmann *et al.*, 2013; Measey and Schweitzer-Stenner, 2011; Sarroukh *et al.*, 2013; Tycko, 2006, 2011; Williams and Pukala, 2013) or fibril pathway of a particular disease (Pryor *et al.*, 2012). In this review we have given an overview of various techniques employed so far to study different species of proteins which are prone to aggregation.

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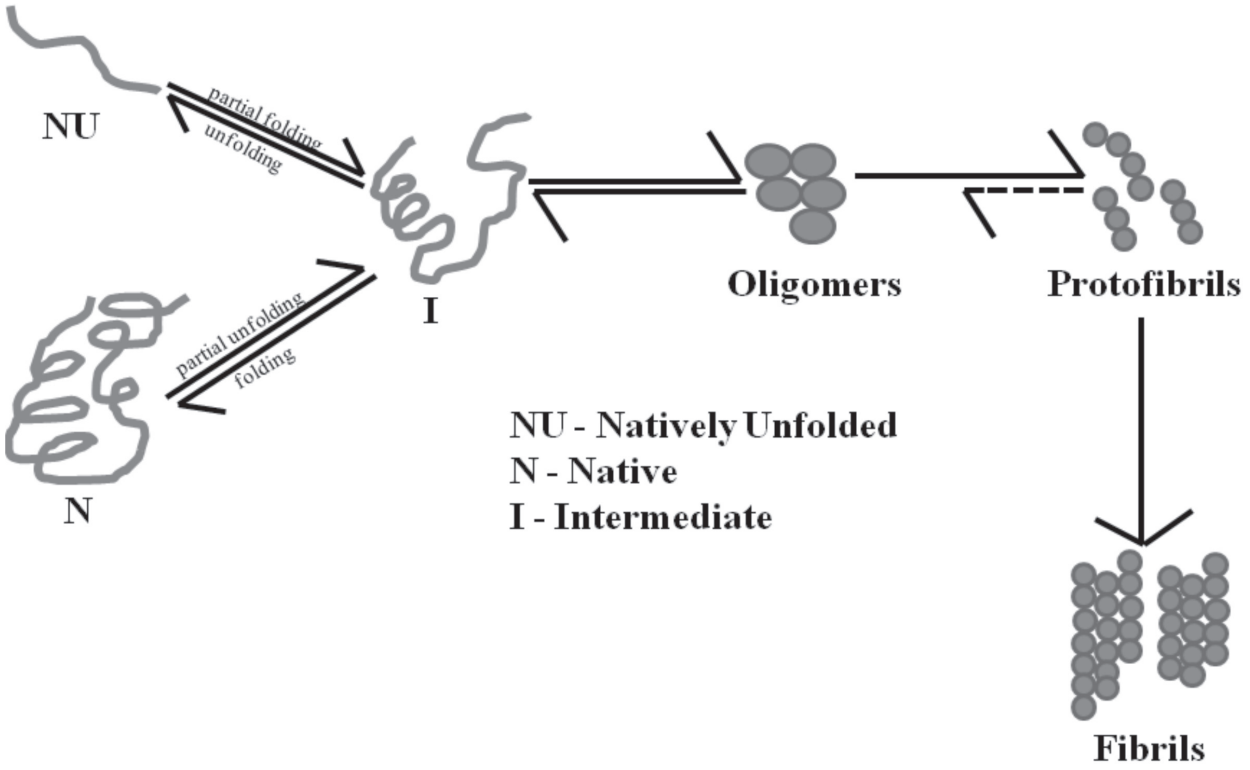

Scheme 1: **A schematic representation of amyloid fibril formation pathway. A variety of precursors such as intermediates obtained from folding/unfolding of native proteins, oligomers, protofibrils exist in fibril formation pathway**

2. Characterization of Conformational change in Partially Folded Forms

Intermediates obtained either by partial unfolding of native proteins or by partial folding of natively unfolded proteins have been suggested to be involved in amyloid fibril formation (Dobson, 2003; Eisenberg *et al.*, 2006; Fink, 1998) and the details of protein folding pathways has been reviewed elsewhere (Ahluwalia *et al.*, 2012).

2.1. Spectroscopic Analysis

Spectroscopic techniques such as Fourier Transform Infrared Spectroscopy (FTIR) and Circular Dichroism (CD) spectroscopy are extensively used to monitor the structural features of the intermediates *in-vitro*. FTIR is a non-destructive technique and can be used under wide variety of conditions (Sarroukh *et al.*, 2013). Nine characteristic IR absorption bands has been observed for polypeptides but the band in the range of 1600-1700 cm^{-1} is most sensitive to protein conformational changes and is referred as amide I band. This spectral region corresponds mainly to C=O vibrations of protein backbone. Each secondary structural element (alpha helix, beta sheet etc.) gives a characteristic peak in the amide I region in aqueous solutions (Kong and Yu, 2007) (Table 1) and can be used to obtain the quantitative information about them. Isotope edited FTIR method has been employed to assign secondary structures at amino acid level (Silva *et al.*, 2003).

Table 1
Characteristic IR bands of secondary structure element of a protein

Secondary structural element	*Wavenumber (cm^{-1})*
β – helix	1654 -1658
β – sheet	1624 -1642
β – turn	1688, 1680, 1672, 1666
Random Coil	1648 ± 2

CD spectra of intermediates can tell us about their structural features. There are mainly three chromophores present in proteins which give rise to CD absorption spectra: (1) peptide bond which absorb in far-UV region i.e. 190 – 240 nm (2) aromatic amino acid side chains show absorption

in near–UV region i.e. between 260- 320 nm (3) disulphide bond show absorption at ~ 260 nm. Far-UV CD spectroscopy is generally applied to monitor the secondary structural elements of proteins. Each type of secondary structural component gives a characteristic spectrum in Far-UV region. a-helix exhibits two distinct bands at 208 and 222 nm whereas an absorption minimum at 215-218 nm has been observed for β-sheet conformation. Random coil conformations are characterized by a maximum at 212 nm and minimum at 196 nm. Different algorithms like SELCON (Sreerama and Woody, 1993), VARSLC (Manavalan and Johnson, 1987), CDSSTR (Johnson, 1999), CONTIN (Provencher and Glockner, 1981) can be used to extract the quantitative estimation of secondary structural element from Far-UV CD data. Near-UV CD is invaluable tool to visualise small conformational changes occurring in proteins as aromatic amino acid side chains are very sensitive to their environment.

Change in tryptophan fluorescence (due to its high quantum yield) can be used to understand the change around tryptophan residues (Saini and Deep, 2010) whereas extrinsic probes like ANS, bis-ANS, Nile Red etc. can be used to monitor the changes in surface hydrophobicity during early events of protein aggregation (Hawe *et al.*, 2008; Lindgren *et al.*, 2005). Fluorescence Resonance energy transfer (FRET) can be applied to determine intra and inter molecular distances and therefore can provide useful information regarding intermediates (Li *et al.*, 2009).

2.2. Structural Characterization

Solution state NMR may be used to understand structural features of the intermediates and can also be used to determine the structure. Chemical shift perturbation can tell us about the changes happening at the residue level (Wishart *et al.*, 1991), changes in J-coupling constants can describe the modulation in torsion angle of correlated atoms in the intermediate (Cornilescu *et al.*, 1999), changes in NOE signal intensity can give information about the change in distance between two correlated atoms. Thus, these measurements can provide meaningful information about changes in secondary and tertiary structure during monomer to intermediate transition.

Raman Optical Activity (ROA) has been developed as a powerful tool to probe protein structure. It is a vibrational spectroscopic technique which measures difference in intensity of Raman scattered right and left circularly polarised light and is very sensitive to chirality (Barron *et al.*, 2000). Specific ROA band has been assigned to secondary structure elements in protein. A broad positive band at 1310 cm^{-1}, due to $^{\alpha}$CH, NH deformation and $^{\alpha}$CH stretching, has been assigned to a-helix whereas a sharp positive and negative ROA couplet of amide I at higher frequency has been assigned to b-sheet. A ROA band at ~1340 cm^{-1} has been assigned to hydrated a-helix (Yamamoto, 2012). Two dimensional correlation analysis of ROA indicated that aggregation of poly (L-lysine) is preceded by intramolecular conversion of α- helix to β- sheet (Ashtony *et al.*, 2006).

Deep Ultraviolet resonance Raman (DUVRR) spectroscopy has recently emerged as a very useful technique for protein structural characterization of different fibrillogenic species formed during amyloid formation (Lednev *et al.*, 2005). Xu *et al.* employed advanced statistical techniques like 2D-correlation spectroscopy, independent component analysis (ICA), and pure variable methods to characterize early stages of fibrillation of lysozyme using DUVRR (Xu *et al.*, 2007).

Molecular dynamic (MD) simulations at high temperature can be used to look at the structure of the intermediates (Wang *et al.*, 2013). Several studies on proteins have found interesting correlation between structural changes observed during simulations at high temperature and experimental data related to *in-vitro* or *in-vivo* aggregation. Table 2 lists certain recent examples of various techniques employed to characterize partially folded forms of proteins during aggregation pathway.

3. Characterization of oligomers and proto-fibrils

3.1. Spectroscopic Analysis

FTIR has also become very useful technique to study the precursors of fibril with the

Table 2
List of some proteins whose intermediates in the fibril formation pathway have been characterized along with techniques used for their characterization

Protein	*Technique Used*	*Information*
Superoxide Dismutase 1 (Leal *et al.*, 2013)	ATR-FTIR, far UV CD, ANS binding assay	Conformational changes during Ca^{2+} modulated aggregation
Prion protein (Serpa *et al.*, 2013)	Limited proteolysis, chemical cross linking, H/D exchange kinetics.	Major conformational change between the native and oligomeric prion protein forms
α- synuclein (Pivato *et al.*, 2012)	FTIR	Secondary structural content in protein aggregates
	Far-UV CD	Structural transition from random coil to α helix
α-synuclein (Kang *et al.*, 2013)	NMR	Monomer transient secondary structure
α-synuclein (Camilloni and Vendruscolo, 2013)	NMR	Relationship between aggregation and secondary structure populations
β2 microglobulin (Yanagi *et al.*, 2012)	NMR	Monomer seed interaction mechanism leading to fibrillation
Bovine serum Albumin (Sharma *et al.*, 2010a; Sharma *et al.*, 2010b)	Trp, Nile red & ANS florescence, Far-UV CD, proteolysis.	Conformation of intermediate
Acylophosphatase (Chong *et al.*, 2011)	MD simulation	Effect of mutation on conformational change
Immunoglobulin light chain (Nowak, 2004)	MD simulation	Conformational behaviour
Transforming Growth Factor-b (Nayeem and Deep, 2010)	MD simulation	Conformation of intermediate, Unfolding of H3-helix
Transthyretin (Rodrigues *et al.*, 2010)	MD simulation	Conformations having high potential for amyloid formation
Insulin (Yamamoto and Watarai, 2012)	ROA	Prefibrillar intermediates
Human Lysozyme (Blanch *et al.*, 2000)	ROA	Prefibrillar intermediates
Hen egg white lysozyme (Xu *et al.*, 2008)	DUVRR, Far UV CD, Trp fluorescence	Parially folded intermediates

development of Attenuated Total Internal Reflectance (ATR) method. ATR – FTIR has an advantage of handling the insoluble aggregates (Sarroukh *et al.*, 2013). It has been observed from various studies that protein oligomers mainly adopt antiparallel- β sheet conformation with a characteristic high frequency band (1685 – 1695 cm^{-1}) and low frequency band (~1630 cm^{-1}).

Some of the dyes like dicyanovinyl julolidine (DCVJ) have been used to probe the early events of oligomerization process and time resolved fluorescence anisotropy decay of ANS gave insights into the structure and kinetics of oligomers (Lindgren *et al.*, 2005). Orte and co workers have reported the characterization of amyloidogenic oligomers by single molecular fluorescence spectroscopy (Orte *et al.*, 2008). Fluorescence correlation spectroscopy (FCS) measures the temporal fluctuations of the fluorescence intensity from dye labelled molecules arising due to their motion from small excitation volume (Hoffmann *et al.*, 2013) and therefore have been employed to determine the number, diffusion time and size of oligomers (Matsumura *et al.*, 2011).

Single Molecule Assays (Single Molecule Fluorescence, Single Molecule Force Microscopy and Nano Pore analysis) are very powerful tools to map the oligomers at single molecule level during protein folding and aggregation and overcome the challenges of averaging over the ensemble of states (Hoffmann *et al.*, 2013). Orte *et*

al. have reported the characterization of amyloidogenic oligomers of bovine phosphatidylinositol-3-kinase (PI3–SH3) (Orte *et al.*, 2008). Trexler and Rhoades have very nicely reviewed the application of single molecule fluorescence methods for probing conformational changes and aggregation of á synuclein (Trexler and Rhoades, 2013).

Fluorescence imaging techniques have been also reported to give clear images of protein aggregates at early stages. Nile Red Fluorescence Microscopy allows detecting aggregates at early stages and also any subtle changes occurring in the aggregates states (Demeule *et al.*, 2007). The images obtained by confocal microscopy have better resolution as compared to those obtained from conventional fluorescence microscopy because out of focus signal is eliminated in confocal microscope by using point illumination and pin-hole aperture. Self assembly process of two oppositely charged proteins and characterization of spherical aggregates formed during this process has been recently reported by Salvatore and co-workers by using confocal laser scanning microscopy (CLSM) (Salvatore *et al.*, 2011).

3.2. Particle Size Distribution

Electrophoretic methods (SDS-PAGE and native-PAGE) allows qualitative and semi quantitative determination of oligomeric distribution of proteins. SDS-PAGE is commonly used to monitor the size distribution of oligomers, however, it is not suitable for non-covalent aggregation since sodium dodecyl sulphate (SDS) dissociates these aggregates. The limitation of SDS-PAGE can be overcome by native-PAGE as SDS is not used in this method. The electrophoretic mobility of proteins in native-PAGE depends on both electric charge and hydrodynamic radius. The details of limitations and applications to protein aggregation have been reviewed elsewhere (Li *et al.*, 2009; Pryor *et al.*, 2012).

Size Exclusion chromatography (SEC) is also a complementary tool to electrophoretic methods for estimation of size of oligomeric species. SEC can be utilized for the separation of monomeric and various oligomeric forms of proteins. Recently SEC was employed to separate oligomers from monomeric α-synuclein in order to study the binding of curcumin to oligomers (Singh *et al.*, 2013).

Photo induced cross linking of unmodified proteins (PICUP) has been extensively used to capture size distribution of metastable oligomer or pre-fibrils. PICUP stabilises these metastable oligomer populations and with the aid of SEC or SDS-PAGE provide snapshots of these species. The advantages of this technique over other conventional cross linking techniques are that neither spacers are used nor pre facto chemical modification of native sequence is required. Moreover, there is only short exposure of non destructive visible light (Bitan, 2006). Different aspects of metastable amyloid and oligomeric assemblies of Aβ peptide has been extensively studied by using this approach (Bitan *et al.*, 2003; Bitan *et al.*, 2001). PICUP has also been applied to reveal early events of oligomerization of α synuclein (Li *et al.*, 2006).

Analytical Ultra centrifugation (AUC) method has wide range of applications to study various proteins. AUC has two modes of measurements: Sedimentation velocity (SV) and sendimentation equilibrium (SE). Analytical centrifuge are equipped with any of the three optical systems i.e. absorbance, interference or fluorescence to carry out the analysis (Cole *et al.*, 2008). SV method is more sensitive to heterogeneity of solutions and therefore commonly used for characterization of protein aggregation. This method is complementary to SEC technique without some of the inherent limitation associated with SEC. Mok et al (Mok *et al.*, 2011) have elaborately reviewed the details of SV using fluorescence detection method and its applications to resolve protein aggregates.

Dynamic light scattering (DLS) studies can be carried out to determine particle size distribution in aggregated protein samples. DLS measures the temporal fluctuations of the intensity of light scattered from particles present in protein samples which are related to the rate of diffusion and hence to hydrodynamic radius.

3.3. Structural Characterization

Limited proteolysis has also become a valuable tool to determine solvent exposed, flexible and

rigid regions in various states of protein. Commonly used proteases are trypsin and pepsin. Trypsin cleaves peptide chains mainly at C-terminal ends of amino acids lysine and arginine (except when followed by proline residues) and pepsin cleaves the peptide chain between hydrophobic and preferably aromatic amino acids. Recently, Serpa *et al.* have performed limited proteolysis using both trypsin and pepsin for characterization of structural differences between native and aggregated β oligomeric forms of proteins (Serpa *et al.*, 2013).

Ishii and co workers (Chimon and Ishii, 2005; Chimon *et al.*, 2007) utilized solid state NMR (ssNMR) for characterization of spherical oligomers of $A\beta_{1"40}$ and their results indicated that $A\hat{a}_{1"40}$ fibrils and oligomers exhibit similar molecular conformations. Site directed spin labelling (SDSL) EPR has been applied for carrying out structural and dynamics studies of oligomers (Akoury *et al.*, 2013).

Dark-state exchange saturation transfer NMR spectroscopy (DEST) is recently proposed by Fawzi *et al.* (Fawzi *et al.*, 2011) for characterizing the interaction between free-state (NMR visible) Ab monomer and a dark or NMR-visible state transiently bound to a high molecular weight species like proto-fibrils. When a molecule binds to a high molecular weight species, it becomes NMR-invisible due to marked increase in transverse relaxation rate that are order of magnitude larger than those of the free monomer. DEST exploits the effect of far off-resonance weak RF field on free and bound state (Fawzi *et al.*, 2012). The bound state resonances, in contrast to free-state, can be partially saturated by far-off-resonance field due to their large R_2 value. The saturation of resonances of bound state is transferred back to resonances in free-state by chemical exchange leading to the attenuation in the signal of observable resonances of free-state. The decrease in NMR signal as a function of RF offset is dependent on R_2 of the resonances of bound-state. Thus, the difference in R_2 of a sample containing a bound state and a reference sample containing only free-state will enable the dynamics in the bound state to be probed on a residue-by-residue basis.

Electron paramagnetic resonance (EPR) has been applied to follow protein dynamics, molecular orientation, ligand binding. This technique is well suited to study protein folding, oligomerization and fibril formation. Site Directed Spin Labelling (SDSL) EPR has been exploited to probe local structure of individual residues. In SDSL method a cysteine residue at desired site is introduced and is labelled with EPR detectable probe (Klug and Feix, 2008). Intramolecular distance of monomers can be determined by using this technique (Murakami *et al.*, 2007).

Ab initio modelling of data obtained from small angle scattering techniques, such as SANS or SAXS, can be used to obtain low-resolution three-dimensional structure of oligomers (Langkilde and Vestergaard, 2009). Vestergaard *et al.* were able to describe low resolution structure of oligomeric structural nucleus of insulin using *ab initio* modelling based on the SAXS data (Vestergaard *et al.*, 2007). Similarly, Oliveira *et al.* were able to describe the structure of oligomeric species of glucagon using SAXS data (Oliveira *et al.*, 2009). A pre-fibrillar heptamer is predicted to exist during early stages of a-synuclein fibrillation using SAXS/SANS data (Tashiro *et al.*, 2008).

Ion mobility spectrometry-Mass spectrometry (IMS- MS) has been widely used to investigate different protein conformers during amyloid formation. In this technique the ions of the samples are generated in gas phase by using various ionization methods like soft ionization methods (e.g. Electron spray ionization) or laser desorption/ ionization methods (e.g. MALDI) (Williams and Pukala, 2013). Smith and co-workers have utilised ESI-IMS-MS for structural characterization of oligomers of â2-microglobulin during fibrillation. (Smith *et al.*, 2010). Table 3 illustrates some selective recent examples of techniques applied to characterize oligomeric species formed during protein fibrillation process.

4. Characterization of Amyloid Fibrils

4.1. Spectroscopic analysis

It has been demonstrated that in FTIR only low frequency band appears (1626 – 1630 cm^{-1}) corresponding to amyloid fibrils and is due to the contribution from parallel β-sheet conformation

Table 3
List of some proteins whose oligomers in their fibril formation pathway have been characterized, along with techniques used for characterization

Protein	*Technique Used*	*Information*
S100A6 (Botelho *et al.*, 2012)	ATR FTIR	Oligomers
Aβ peptide (Cerf *et al.*, 2009)	ATR FTIR	Oligomers
Aβ 42 (Gu *et al.*, 2013)	SDSL EPR	Oligomer structure
Tau protein (Akoury *et al.*, 2013)	SDSL EPR	Oligomer structure and dynamics
SH3 domain of α-spectrin (Paredes *et al.*, 2012)	Fluorescence lifetime correlation spectroscopy (FLCS)	Size distribution and kinetics of oligomer growth
Aβ 42 (Jeong *et al.*, 2013)	Ex-situ and in-situ AFM	Oligomers
α synuclein (Ahmad and Lapidus, 2012)	Far UV CD	Oligomers (partially folded monomer to β sheet structured aggregate)
α synuclein (Singh *et al.*, 2013)	SEC, HSQC NMR, Far-UV CD, AFM	Oligomers
α crystalline (Smirnova *et al.*, 2013)	SDS and Native PAGE, SEC, DLS, AUC	Flexible oligomeric structure
α-synulein (Nath *et al.*, 2010)	FCS, FRET	Oligomer, conformational changes during oligomer formation.
Tau Protein (Bader *et al.*, 2011)	FCS	Oligomer
α synuclein (Ono *et al.*, 2011)	PIUCP, AFM, EM,	Oligomers
α synuclein (Kaylor *et al.*, 2005)	FRET, Trp fluorescence, FTIR, DLS	Transient oligomers
Murine amylin (Palmieri *et al.*, 2013)	ESI-IMS-MS	Oligomeric size distribution
Aβ 42 (Bernstein *et al.*, 2005)	IMS-MS	Oligomeric size distribution
Insulin (Vestergaard *et al.*, 2007)	SAXS	Oligomer and repeating unit of fibril.
Glucagon (Oliveira *et al.*, 2009)	SAXS	Oligomer structure
α-synuclein (Tashiro *et al.*, 2008)	SAXS	Oligomer structure

(Sarroukh *et al.*, 2013). Linearly polarized ATR-FTIR method and two-dimensional IR correlation spectroscopy may provide detailed information of protein structure and orientation.

Vibrational Circular dichroism (VCD) is an extension of circular dichroism in infrared and near infrared regions. Recently evidences have emerged that VCD is sensitive to the presence of amyloid fibrils. Enhancement in the intensity of the amide I band of VCD spectra have been demonstrated (Ma *et al.*, 2007). Moreover details of supramolecular architecture of fibrils can also be obtained from VCD (Ma *et al.*, 2007).

Frare and co-workers have used limited proteolysis in combination with Mass Spectrometry analysis to decipher the core structure of lysozyme fibrils (Smith *et al.*, 2010). Similarly Monti *et al* have utilised this approach to discriminate between the inaccessible core and flexible, solvent exposed regions of fibrils of β2-microglobulin (β2-m) and truncated ΔN6β2-m (Monti *et al.*, 2005).

Thioflavin T (ThT) is one of the commonly used fluorescent dyes for characterization of amyloid fibrils as it shows significant increase in fluorescence emission on binding to fibrils. But in some cases ThT has also shown response to early oligomers (Hurshman *et al.*, 2004). Thioflavin S is another dye which can be used to monitor fibrils and they do not bind to monomers (Li *et al.*, 2009). Congo red binding and birefringence is complementary to ThT assay to follow amyloid formation. Interaction of amyloid

and congo red leads to shift in UV absorbance of congo red from 490 to 540 nm. Congo red also yields apple green birefringence under cross-polarized light in the presence of amyloid fibrils (Hawe *et al.*, 2008). Dyamics of amyloid fibrils have been recently reported by using FRET (Belitzky *et al.*, 2011).

4.2. Structural characterization

X-ray diffraction pattern of fibril had provided important information about structural features of amyloid. The diffraction pattern obtained from amyloid fibril display very strong intensities corresponding to distance of 4.7 Å in meridian plane and 10-11 Å in meridonal plane in real space. These distances are typical distance observed for β-sheet sandwich suggesting a common structural motif for all amyloids i.e. a spine formed by extensive β-sheets with individual β-strand perpendicular to it (Langkilde and Vestergaard, 2009).

Due to inherent problems associated with other techniques and with the improvement in methodologies, solid state NMR spectroscopy is becoming the method of choice for the structure determination of amyloid (Tycko, 2011). Full molecular structural models have been developed for amyloids of various proteins using the constraints obtained from solid-state NMR supplemented by information obtained from electron microscopy. The constraint on backbone conformation comes from measurement of isotropic ^{13}C and ^{15}N NMR chemical shift. There is a strong correlation between the chemical shift of ^{13}C of CO, C_α, C_β and ^{15}N and secondary structure in proteins (Wishart *et al.*, 1991). There is also a correlation between C_α, C_β chemical shift with backbone torsion angle φ and ψ (Cornilescu *et al.*, 1999). Similarly, line width measurements also enable us to provide constraints on the peptide backbone. For conformationally ordered peptide in rigid non-crystalline environment, the ^{13}C NMR line-width is in 1.5-2.5 ppm range whereas conformational disorder is indicated by higher linewidth (Tycko, 2006). A microcrystalline or nearly crystalline system has line-width much smaller than 1.5 ppm. Solid-state NMR spectra of spin-1/2 nuclei are not only determined by chemical shifts as in solution NMR, but also by chemical shift anisotropy (CSA) and dipolar-dipolar couplings since they are not averaged to zero due to absence of isotropic tumbling. Rapid Magic angle spinning (MAS) is necessary to average both CSA and dipolar coupling to zero. Another important constraint comes from the measurement of dipole-dipole coupling since it is proportional to the inverse cube of internuclear distance and gyromagnetic ratios of the ratios on both spins. Since rapid MAS averages the dipole-dipole coupling to zero, special pulse sequence techniques called 'dipolar recoupling' have been developed to produce non-zero time-averaged dipole-dipole coupling under MAS (Gregory *et al.*, 1995; Gullion and Vega, 1992; Meier and Earl, 1986; Tycko, 2006).

Structural models obtained from solid state NMR have shown polymorphic nature of amyloid fibrils which may happen due to variation in nature of β-sheet structure and symmetry (Petkova *et al.*, 2005). Solid state NMR studies have shown that in-register parallel β-sheet structures are most common in amyloid and prion fibrils, but antiparallel and β-helical structures have also been reported (Tycko, 2011). Full structural models for striated-ribbon and twisted pair filaments of $A\beta_{1-40}$ have been obtained from backbone torsion angle constraints, inter and intra dipolar coupling, and semiquantitative measurement of intramolecular ^{13}C-^{13}C distance measurement (Petkova *et al.*, 2005; Petkova *et al.*, 2006; Tycko, 2011).

EPR has become well established method to investigate structural features of fibrils (Chen *et al.*, 2007; Cobb *et al.*, 2007; Jayasinghe and Langen, 2004) and intermolecular distance analysis (Torok *et al.*, 2002).

4.3. Microscopic Analysis

Electron microscopic (EM) techniques have emerged as very powerful tools to characterize and visualize the morphology of fibrils formed during aggregation process. Both Scanning Electron Microscopy (SEM) and Transmission electron microscopy (TEM) are low resolution method but have huge advantage of being a direct method (Langkilde and Vestergaard, 2009). With the advent of cryo-TEM and advances in reconstruction method, it is possible to get high resolution structure for fibrils. Meinhardt *et al.*

Table 4
List of some proteins whose protofibrils/fibrils in their fibril formation pathway have been characterized along with techniques used for characterization

Protein	*Technique Used*	*Information*
Aβ peptide (Cerf *et al.*, 2009)	ATR FTIR	Fibrils
Aβ peptides (Shivu *et al.*, 2013)	ATR-FTIR	Amyloid fibrils
Aβ peptide (1-40) (Belitzky *et al.*, 2011)	FRET, confocal microscopy	Dynamics and morphology of amyloid fibrils
α-synuclein (Pornsuwan *et al.*, 2013)	PELDOR EPR	Long range distances in fibrils
Aβ 40 and Aβ 42 (Gu and Guo, 2013)	SDSL EPR	Interactions between Aβ 40 and Aβ 42 in interlaced fibrils
α-synuclein (Der-Sarkissian *et al.*, 2003)	EPR	Structural organization of fibril
TTR (Fitzpatrick *et al.*, 2013)	NMR	Atomic structure of cross β protofilaments and fibrils
Aβ (Petkova *et al.*, 2006)	ssNMR	Fibril
Ure-2p (10-39) (Chan *et al.*, 2005)	ssNMR	Fibril
TTR (Jaroniec *et al.*, 2002; Jaroniec *et al.*, 2004)	ssNMR	Molecular conformation of peptide fragment in amyloid fibril
Islet amyloid polypeptide (Jayasinghe and Langen, 2004)	SDSL-EPR	Structural features of fibril
α-synuclein (Heise *et al.*, 2005)	ssNMR	Molecular level structure, polymorphism and dynamics of Fibril.
Tau protein (Inoue *et al.*, 2012)	X ray Diffraction, FTIR, TEM	Fibrils
TTR1 (Bongiovanni *et al.*, 2012)	Cryo TEM, X ray diffraction	Fibrils
α, β, γ crystallin (Meehan *et al.*, 2004)	X ray diffraction, TEM, Congo Red, ThT assay	Fibrils
Ovalbumin (Lara *et al.*, 2012)	AFM, SLS, DLS, SAXS	Kinetics of fibril growth
Murine amylin (Palmieri *et al.*, 2013)	X-ray Diffraction, FTIR, AFM, TEM	Fibrils
TTR (Cardoso *et al.*, 2002)	STEM, TEM , AFM	Fibrils
SOD1(Leal *et al.*, 2013)	TEM, ThT fluorescence	Fibrils
Aβ 42 (Jeong *et al.*, 2013)	Ex-situ and in-situ AFM	Protofibrils and fibrils
β2- microglobulin (Yoshimura *et al.*, 2012)	TEM, AFM, ThT fluorescence	Amyloid fibrils
SOD1 (Oztug Durer *et al.*, 2009)	Congo Red birefringence, ThT fluorescence, EM, AFM	Amyloid fibrils
HSA, OVA (Naeem and Amani, 2013)	SEM	Fibrils
(AT3Q24) ataxin-3, an expanded (AT3Q55) ataxin-3 (Natalello *et al.*, 2011)	H-D exchange/FTIR	Glutamine side chain hydrogen bonding key feature of irreversible aggregates (fibrils)
alanine-rich octamer, Ac-(AAAAKAAY)-NH2 (AKY8) (Measey and Schweitzer-Stenner, 2011)	VCD	Amyloid like fibrils
Poly Q peptides (Kurouski *et al.*, 2013)	VCD, DUVRR	Supramolecular organization of fibrils.
β2 microglobulin (Yoshimura *et al.*, 2010)	Solution State NMR	Conformation and flexibility of amyloid fibrils
Insulin (Yamamoto and Watarai, 2012)	ROA	Amyloid fibril
α synuclein (Lee *et al.*, 2009)	JC-1 fluorescent probe, FRET	Amyloid fibrils
β2 microglobulin (Myers *et al.*, 2006)	Limited proteolysis	Conformational prooerties of in vitro fibrils of different morphologies
SOD1 (Chan *et al.*, 2013)	Limited proteolysis, AFM	Structural similarity between fibrils of wild type and mutant SOD1
Lysozyme (Shashilov *et al.*, 2007)	DUVRR	Secondary structure of fibril cross β core
Aβ 40 and $A\beta_{34\text{-}42}$ (Popova *et al.*, 2010)	DUVRR	Fibril core

Table 5
Advantages and disadvantages/limitations of various techniques used to understand the structural features of species in amyloid formation pathway

Technique	*Advantages*	*Disadvantages/ Limitations*
ATR-FTIR	Less time and sample required. Secondary structure of intermediates or fibrils can also be obtained as IR is insensitive to light scattering.	Residue level information cannot be obtained.
Isotope Edited FTIR	Structure determination of amyloid at amino acid level.	Structure can only be obtained around labelled residue.
Linear Dichroism IR	Orientation of fibrils can be obtained.	Not suitable for oligomers due to their anisotropy.
CD spectroscopy	Convenient tool to monitor secondary and tertiary structural changes during aggregation process. Quantitative estimation of secondary structures in different intermediates.	Structure at residue level can not be obtained. Scattering from aggregate may interfere in the measurement.
Fluorescence spectroscopy	Convenient tool, rapid data acquisition, high signal to noise ratio, sensitive, less sample required.	
(a) Intrinsic fluorescence	Can monitor structural changes around tryptophan or tyrosine during early events.	Can not give information about secondary level structure or structure at residue level.
(b) Extrinsic fluorescence	Dyes binding specifically to particular species can provide information regarding structural features of intermediates, oligomers or fibrils involved.	Sometimes misleading. Dyes may interfere with the aggregation process. Dye may not bind specifically to a particular species.
© Single molecule fluorescence spectroscopy	Overcome the problem of ensemble averaging. Characterization of subpopulations in heterogenous ensemble is possible.	Sophisticated instrumentation required. Biochemical modifications required in certain assays. Can not tell about secondary and tertiary structure changes.
(d) Fluorescence correlation spectroscopy (FCS)	Higly sensitive, rapid, wide range of particle sizes can be analysed	Labelling of protein is required. No detailed structural information.
(e) FRET	Intermolecular and intramolecular distance between acceptor and donor groups.	Only qualitative information obtained in case of aggregates as FRET efficiency varies with different orientations in different aggregates.
ROA **VCD**	Secondary structure of different species in amyloid formation pathway. Better resolution than conventional vibrational and raman spectroscopy.	No structural information at residue level.
Electron Microscopy (SEM, TEM)	Direct visualization of morphology of aggregates	Low resolution technique. No structural information.
SDS PAGE	Oligomers distribution on the basis of size and shape.	No structural information. Not suitable for non covalent aggregates. SDS may result in non native behaviour. Gel smearing may interfere with any conclusive finding.
Native PAGE	Oligomers separation on the basis of size and charge. Native conditions intact.	No structural information. Gel smearing may interfere with any conclusive finding.
PICUP	Quantitative snapshots of size of oligomers. No spacer required. No chemical modification required therefore analysis can be done in intact state of proteins.	No structural information. No information about size of fibrils.

SEC	Separation of oligomers. Oligomer size distribution profile.	Not suitable for fibrils. No structural information.
X Ray diffraction	Static snapshot of fibrils. Atomic level structure can be obtained.	Large amount of protein required. Crystallisation of protein aggregates required. Degree of order of amyloid fibrils is not high enough to obtain high resolution structure.
Solution state NMR	High resolution structure can be obtained of soluble species in amyloid formation pathway. Suitable of oligomers.	Large amount of protein required. Labelling of protein is needed. Not suitable for fast structural changes. Heterogeneous nature of oligomers may make studying such oligomers a difficult task.
Solid state NMR	High resolution structure of amyloid fibrils.	High protein concentration and labelling of protein is needed. Not suitable for fibrils of protein with high molecular weight.
ESR	High time scale resolution and high signal to noise ratio. Monitor protein molecular dynamics during protein oligomerization and fibrillation.	Spin Labelling is required. Low resolution method.
IMS-MS	Changes in Structural features during aggregation process.	Low resolution method.
DLS	Oligomer size distribution profile of heterogenous samples.	Scattering depends exponentially on aggregate size. Does not give informational about changes in structural features.
AUC	Wide range of aggregate sizes can be analyzed.	Theoretical size approximation requires appropriate assumptions in the model. . Does not give informational about changes in structural features.

have obtained 12 different morphologies from a single sample of Aβ (Meinhardt *et al.*, 2009). In contrast to EM techniques, Scanning Tunnelling Microscopy (STEM), and Atomic Force microscopy (AFM) produce single particle 2D profile. Smaller particle can be easily observed and may be used to visualize on- and off-pathway oligomer (Ding *et al.*, 2002; Losic *et al.*, 2006). Belitzky et al have demonstrated the dynamics of Aβ (1-40) fibrils by using Confocal microscopy in combination with FRET (Belitzky *et al.*, 2011).

Some of the examples of recent developments in diagonistic tools for characterization of fibrils have been listed in Table 4.

There are varieties of techniques available to understand and determine the structure of the fibrils and their precursors. However, each of the techniques has its limitation. Table 5 describes the advantages and disadvantages/limitations of various techniques. It is imperative that data must be obtained from complementary techniques to obtain a precise picture of the aggregation pathway.

5. Summary

This review has discussed the techniques used currently by the researchers. A lot of advancement in techniques has been made which makes us possible to understand the mechanism of the amyloid formation. However, much needs to be done to understand what drives the amyloid formation. Nevertheless, this review will help one to decide the right technique to be used for a specific problem, provide a comprehensive view of the available techniques to the uninitiated and help obtain a landscape of the current literature as well.

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Abbreviations

FTIR, Fourier Transform Infrared Spectroscopy; CD, circular Dichroism; FRET, Fluorescence Resonance Energy Transfer; ROA, Raman Optical Activity; DUVRR, Deep Ultraviolet resonance Raman; MD, Molecular dynamic; ATR, Attenuated Total Internal Reflectance ; DCVJ, dicyanovinyl julolidine; FCS, Fluorescence correlation spectroscopy; SEC, Size Exclusion chromatography; PICUP, Photo induced cross linking of unmodified proteins; AUC, Analytical Ultra centrifugation; SV, Sedimentation velocity; SE, Sedimentation equilibrium; DLS, Dynamic light scattering ; ssNMR, solid state NMR; SDSL EPR, Site directed spin labelling electron paramagnetic resonance; DEST, Dark-state exchange saturation transfer; SAXS, Small angle X ray

scattering; SANS, Small angle neutron scattering; IMS-MS, Ion mobility spectrometry-Mass spectrometry; VCD, Vibrational Circular dichroism; ThT, Thioflavin T; EM, Electron microscopy; SEM, Scanning Electron Microscopy; TEM, Transmission electron microscopy; STEM, Scanning Tunneling Microscopy; AFM, Atomic Force microscopy.

References

Ahluwalia, U., Katyal, N., and Deep, S. (2012). Models of Protein Folding. J Proteins Proteomics *3*, 85-93.

Ahmad, B., and Lapidus, L.J. (2012). Curcumin prevents aggregation in alpha-synuclein by increasing reconfiguration rate. J Biol Chem *287*, 9193-9199.

Akoury, E., Gajda, M., Pickhardt, M., Biernat, J., Soraya, P., Griesinger, C., Mandelkow, E., and Zweckstetter, M. (2013). Inhibition of tau filament formation by conformational modulation. J Am Chem Soc *135*, 2853-2862.

Ashtony, L., Barronz, L.D., Czarnik-Matusewicz, B., Hecht, L., Hyde, J., and Blanch, E.W. (2006). Two-dimensional correlation analysis of Raman optical activity data on the alpha-helix-to-beta-sheet transition in poly(L-lysine). Mol Phys *104*, 1429-1445.

Bader, B., Nubling, G., Mehle, A., Nobile, S., Kretzschmar, H., and Giese, A. (2011). Single particle analysis of tau oligomer formation induced by metal ions and organic solvents. Biochem Biophys Res Commun *411*, 190-196.

Barron, L.D., Hecht, L., Blanch, E.W., and Bell, A.F. (2000). Solution structure and dynamics of biomolecules from Raman optical activity. Prog Biophys Mol Biol *73*, 1-49.

Belitzky, A., Melamed-Book, N., Weiss, A., and Raviv, U. (2011). The dynamic nature of amyloid beta (1-40) aggregation. Phys Chem Chem Phys *13*, 13809-13814.

Bernstein, S.L., Wyttenbach, T., Baumketner, A., Shea, J.E., Bitan, G., Teplow, D.B., and Bowers, M.T. (2005). Amyloid beta-protein: monomer structure and early aggregation states of Abeta42 and its Pro19 alloform. J Am Chem Soc *127*, 2075-2084.

Bitan, G. (2006). Structural study of metastable amyloidogenic protein oligomers by photo-induced cross-linking of unmodified proteins. Methods Enzymol *413*, 217-236.

Bitan, G., Kirkitadze, M.D., Lomakin, A., Vollers, S.S., Benedek, G.B., and Teplow, D.B. (2003). Amyloid beta -protein (Abeta) assembly: Abeta 40 and Abeta 42 oligomerize through distinct pathways. Proc Natl Acad Sci U S A *100*, 330-335.

Bitan, G., Lomakin, A., and Teplow, D.B. (2001). Amyloid beta-protein oligomerization: prenucleation interactions revealed by photo-induced cross-linking of unmodified proteins. J Biol Chem *276*, 35176-35184.

Blanch, E.W., Morozova-Roche, L.A., Cochran, D.A., Doig, A.J., Hecht, L., and Barron, L.D. (2000). Is polyproline II helix the killer conformation? A Raman optical activity study of the amyloidogenic prefibrillar intermediate of human lysozyme. J Mol Biol *301*, 553-563.

Bongiovanni, M.N., Puri, D., Goldie, K.N., and Gras, S.L. (2012). Noncore residues influence the kinetics of functional TTR(105-115)-based amyloid fibril assembly. J Mol Biol *421*, 256-269.

Botelho, H.M., Leal, S.S., Cardoso, I., Yanamandra, K., Morozova-Roche, L.A., Fritz, G., and Gomes, C.M. (2012). S100A6 amyloid fibril formation is calcium-modulated and enhances superoxide dismutase-1 (SOD1) aggregation. J Biol Chem *287*, 42233-42242.

Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C.M., and Stefani, M. (2002). Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. Nature *416*, 507-511.

Camilloni, C., and Vendruscolo, M. (2013). A Relationship between the Aggregation Rates of alpha-Synuclein Variants and the beta-Sheet Populations in Their Monomeric Forms. J Phys Chem B. In Press.

Cardoso, I., Goldsbury, C.S., Muller, S.A., Olivieri, V., Wirtz, S., Damas, A.M., Aebi, U., and Saraiva, M.J. (2002). Transthyretin fibrillogenesis entails the assembly of monomers: a molecular model for in vitro assembled transthyretin amyloid-like fibrils. J Mol Biol *317*, 683-695.

Cerf, E., Sarroukh, R., Tamamizu-Kato, S., Breydo, L., Derclaye, S., Dufrene, Y.F., Narayanaswami, V., Goormaghtigh, E., Ruysschaert, J.M., and Raussens, V. (2009). Antiparallel beta-sheet: a signature structure of the oligomeric amyloid beta-peptide. Biochem J *421*, 415-423.

Chan, J.C., Oyler, N.A., Yau, W.M., and Tycko, R. (2005). Parallel beta-sheets and polar zippers in amyloid fibrils formed by residues 10-39 of the yeast prion protein Ure2p. Biochemistry *44*, 10669-10680.

Chan, P.K., Chattopadhyay, M., Sharma, S., Souda, P., Gralla, E.B., Borchelt, D.R., Whitelegge, J.P., and Valentine, J.S. (2013). Structural similarity of wild-type and ALS-mutant superoxide dismutase-1 fibrils using limited proteolysis and atomic force microscopy. Proc Natl Acad Sci U S A *110*, 10934-10939.

Chen, M., Margittai, M., Chen, J., and Langen, R. (2007). Investigation of alpha-synuclein fibril structure by site-directed spin labeling. J Biol Chem *282*, 24970-24979.

Chimon, S., and Ishii, Y. (2005). Capturing intermediate structures of Alzheimer's beta-amyloid, Abeta(1-40), by solid-state NMR spectroscopy. J Am Chem Soc *127*, 13472-13473.

Chimon, S., Shaibat, M.A., Jones, C.R., Calero, D.C., Aizezi, B., and Ishii, Y. (2007). Evidence of fibril-like beta-sheet structures in a neurotoxic amyloid intermediate of Alzheimer's beta-amyloid. Nat Struct Mol Biol *14*, 1157-1164.

Chiti, F., and Dobson, C.M. (2006). Protein misfolding, functional amyloid, and human disease. Annu Rev Biochem *75*, 333-366.

Chiti, F., Webster, P., Taddei, N., Clark, A., Stefani, M., Ramponi, G., and Dobson, C.M. (1999). Designing conditions for in vitro formation of amyloid

protofilaments and fibrils. Proc Natl Acad Sci U S A *96*, 3590-3594.

Chong, S.H., Lee, C., Kang, G., Park, M., and Ham, S. (2011). Structural and thermodynamic investigations on the aggregation and folding of acylphosphatase by molecular dynamics simulations and solvation free energy analysis. J Am Chem Soc *133*, 7075-7083.

Cobb, N.J., Sonnichsen, F.D., McHaourab, H., and Surewicz, W.K. (2007). Molecular architecture of human prion protein amyloid: a parallel, in-register beta-structure. Proc Natl Acad Sci U S A *104*, 18946-18951.

Cole, J.L., Lary, J.W., T, P.M., and Laue, T.M. (2008). Analytical ultracentrifugation: sedimentation velocity and sedimentation equilibrium. Methods Cell Biol *84*, 143-179.

Cornilescu, G., Delaglio, F., and Bax, A. (1999). Protein backbone angle restraints from searching a database for chemical shift and sequence homology. J Biomol NMR *13*, 289-302.

Demeule, B., Gurny, R., and Arvinte, T. (2007). Detection and characterization of protein aggregates by fluorescence microscopy. Int J Pharm *329*, 37-45.

Der-Sarkissian, A., Jao, C.C., Chen, J., and Langen, R. (2003). Structural organization of alpha-synuclein fibrils studied by site-directed spin labeling. J Biol Chem *278*, 37530-37535.

Ding, T.T., Lee, S.J., Rochet, J.C., and Lansbury, P.T., Jr. (2002). Annular alpha-synuclein protofibrils are produced when spherical protofibrils are incubated in solution or bound to brain-derived membranes. Biochemistry *41*, 10209-10217.

Dobson, C.M. (2003). Protein folding and misfolding. Nature *426*, 884-890.

Eisenberg, D., Nelson, R., Sawaya, M.R., Balbirnie, M., Sambashivan, S., Ivanova, M.I., Madsen, A.O., and Riekel, C. (2006). The structural biology of protein aggregation diseases: Fundamental questions and some answers. Acc Chem Res *39*, 568-575.

Fawzi, N.L., Ying, J., Ghirlando, R., Torchia, D.A., and Clore, G.M. (2011). Atomic-resolution dynamics on the surface of amyloid-beta protofibrils probed by solution NMR. Nature *480*, 268-272.

Fawzi, N.L., Ying, J., Torchia, D.A., and Clore, G.M. (2012). Probing exchange kinetics and atomic resolution dynamics in high-molecular-weight complexes using dark-state exchange saturation transfer NMR spectroscopy. Nat Protoc *7*, 1523-1533.

Fink, A.L. (1998). Protein aggregation: folding aggregates, inclusion bodies and amyloid. Fold Des *3*, R9-23.

Fitzpatrick, A.W., Debelouchina, G.T., Bayro, M.J., Clare, D.K., Caporini, M.A., Bajaj, V.S., Jaroniec, C.P., Wang, L., Ladizhansky, V., Muller, S.A., *et al.* (2013). Atomic structure and hierarchical assembly of a cross-beta amyloid fibril. Proc Natl Acad Sci U S A *110*, 5468-5473.

Gregory, D.M., Mitchell, D.J., Stringer, J.A., Kiihne, S., Shiels, J.C., Callahan, J., Mehta, M.A., and Drobny, G.P. (1995). Windowless Dipolar Recoupling - the Detection of Weak Dipolar Couplings between Spin-1/2 Nuclei with Large Chemical-Shift Anisotropies. Chem Phys Lett *246*, 654-663.

Gu, L., and Guo, Z. (2013). Alzheimer's Abeta42 and Abeta40 peptides form interlaced amyloid fibrils. J Neurochem *126*, 305-311.

Gu, L., Liu, C., and Guo, Z. (2013). Structural insights into Abeta42 oligomers using site-directed spin labeling. J Biol Chem *288*, 18673-18683.

Gullion, T., and Vega, S. (1992). A Simple Magic Angle Spinning Nmr Experiment for the Dephasing of Rotational Echoes of Dipolar Coupled Homonuclear Spin Pairs. Chem Phys Lett *194*, 423-428.

Hawe, A., Sutter, M., and Jiskoot, W. (2008). Extrinsic fluorescent dyes as tools for protein characterization. Pharm Res *25*, 1487-1499.

Heise, H., Hoyer, W., Becker, S., Andronesi, O.C., Riedel, D., and Baldus, M. (2005). Molecular-level secondary structure, polymorphism, and dynamics of full-length alpha-synuclein fibrils studied by solid-state NMR. Proc Natl Acad Sci U S A *102*, 15871-15876.

Hoffmann, A., Neupane, K., and Woodside, M.T. (2013). Single-molecule assays for investigating protein misfolding and aggregation. Phys Chem Chem Phys *15*, 7934-7948.

Hurshman, A.R., White, J.T., Powers, E.T., and Kelly, J.W. (2004). Transthyretin aggregation under partially denaturing conditions is a downhill polymerization. Biochemistry *43*, 7365-7381.

Inoue, M., Konno, T., Tainaka, K., Nakata, E., Yoshida, H.O., and Morii, T. (2012). Positional effects of phosphorylation on the stability and morphology of tau-related amyloid fibrils. Biochemistry *51*, 1396-1406.

Janson, J., Ashley, R.H., Harrison, D., McIntyre, S., and Butler, P.C. (1999). The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles. Diabetes *48*, 491-498.

Jaroniec, C.P., MacPhee, C.E., Astrof, N.S., Dobson, C.M., and Griffin, R.G. (2002). Molecular conformation of a peptide fragment of transthyretin in an amyloid fibril. Proc Natl Acad Sci U S A *99*, 16748-16753.

Jaroniec, C.P., MacPhee, C.E., Bajaj, V.S., McMahon, M.T., Dobson, C.M., and Griffin, R.G. (2004). High-resolution molecular structure of a peptide in an amyloid fibril determined by magic angle spinning NMR spectroscopy. Proc Natl Acad Sci U S A *101*, 711-716.

Jayasinghe, S.A., and Langen, R. (2004). Identifying structural features of fibrillar islet amyloid polypeptide using site-directed spin labeling. J Biol Chem *279*, 48420-48425.

Jeong, J.S., Ansaloni, A., Mezzenga, R., Lashuel, H.A., and Dietler, G. (2013). Novel mechanistic insight into the molecular basis of amyloid polymorphism and secondary nucleation during amyloid formation. J Mol Biol *425*, 1765-1781.

Johnson, W.C. (1999). Analyzing protein circular dichroism spectra for accurate secondary structures. Proteins *35*, 307-312.

Kang, L., Janowska, M.K., Moriarty, G.M., and Baum, J. (2013). Mechanistic Insight into the Relationship between N-Terminal Acetylation of alpha-Synuclein and Fibril Formation Rates by NMR and Fluorescence. PLoS One *8*, e75018.

Kaylor, J., Bodner, N., Edridge, S., Yamin, G., Hong, D.P., and Fink, A.L. (2005). Characterization of oligomeric intermediates in alpha-synuclein fibrillation: FRET studies of Y125W/Y133F/Y136F alpha-synuclein. J Mol Biol *353*, 357-372.

Klein, W.L., Krafft, G.A., and Finch, C.E. (2001). Targeting small Abeta oligomers: the solution to an Alzheimer's disease conundrum? Trends Neurosci *24*, 219-224.

Klug, C.S., and Feix, J.B. (2008). Methods and applications of site-directed spin labeling EPR spectroscopy. Methods Cell Biol *84*, 617-658.

Kong, J., and Yu, S. (2007). Fourier transform infrared spectroscopic analysis of protein secondary structures. Acta Biochim Biophys Sin (Shanghai) *39*, 549-559.

Kurouski, D., Kar, K., Wetzel, R., Dukor, R.K., Lednev, I.K., and Nafie, L.A. (2013). Levels of supramolecular chirality of polyglutamine aggregates revealed by vibrational circular dichroism. FEBS Lett *587*, 1638-1643.

Langkilde, A.E., and Vestergaard, B. (2009). Methods for structural characterization of prefibrillar intermediates and amyloid fibrils. FEBS Lett *583*, 2600-2609.

Lara, C., Gourdin-Bertin, S., Adamcik, J., Bolisetty, S., and Mezzenga, R. (2012). Self-assembly of ovalbumin into amyloid and non-amyloid fibrils. Biomacromolecules *13*, 4213-4221.

Leal, S.S., Cardoso, I., Valentine, J.S., and Gomes, C.M. (2013). Calcium ions promote superoxide dismutase 1 (SOD1) aggregation into non-fibrillar amyloid: a link to toxic effects of calcium overload in amyotrophic lateral sclerosis (ALS)? J Biol Chem *288*, 25219-25228.

Lednev, I.K., Ermolenkov, V.V., He, W., and Xu, M. (2005). Deep-UV Raman spectrometer tunable between 193 and 205 nm for structural characterization of proteins. Anal Bioanal Chem *381*, 431-437.

Lee, J.H., Lee, I.H., Choe, Y.J., Kang, S., Kim, H.Y., Gai, W.P., Hahn, J.S., and Paik, S.R. (2009). Real-time analysis of amyloid fibril formation of alpha-synuclein using a fibrillation-state-specific fluorescent probe of JC-1. Biochem J *418*, 311-323.

Li, H., Rahimi, F., Sinha, S., Maiti, P., Bitan, G., and Murakami, K. (2009). Amyloid and Protein Aggregation-Analytical Methods. In Encyclopedia of Analytical Chemistry, R.A. Meyers, ed., John Wiley & Sons Ltd., New Jersey, pp. 1-32.

Li, H.T., Lin, X.J., Xie, Y.Y., and Hu, H.Y. (2006). The early events of alpha-synuclein oligomerization revealed by photo-induced cross-linking. Protein Pept Lett *13*, 385-390.

Lindgren, M., Sorgjerd, K., and Hammarstrom, P. (2005). Detection and characterization of aggregates, prefibrillar amyloidogenic oligomers, and protofibrils using fluorescence spectroscopy. Biophys J *88*, 4200-4212.

Losic, D., Martin, L.L., Mechler, A., Aguilar, M.I., and Small, D.H. (2006). High resolution scanning tunnelling microscopy of the beta-amyloid protein (Abeta1-40) of Alzheimer's disease suggests a novel mechanism of oligomer assembly. J Struct Biol *155*, 104-110.

Ma, S., Cao, X., Mak, M., Sadik, A., Walkner, C., Freedman, T.B., Lednev, I.K., Dukor, R.K., and Nafie, L.A. (2007). Vibrational circular dichroism shows unusual sensitivity to protein fibril formation and development in solution. J Am Chem Soc *129*, 12364-12365.

Manavalan, P., and Johnson, W.C., Jr. (1987). Variable selection method improves the prediction of protein secondary structure from circular dichroism spectra. Anal Biochem *167*, 76-85.

Matsumura, S., Shinoda, K., Yamada, M., Yokojima, S., Inoue, M., Ohnishi, T., Shimada, T., Kikuchi, K., Masui, D., Hashimoto, S., *et al.* (2011). Two distinct amyloid beta-protein (Abeta) assembly pathways leading to oligomers and fibrils identified by combined fluorescence correlation spectroscopy, morphology, and toxicity analyses. J Biol Chem *286*, 11555-11562.

Measey, T.J., and Schweitzer-Stenner, R. (2011). Vibrational circular dichroism as a probe of fibrillogenesis: the origin of the anomalous intensity enhancement of amyloid-like fibrils. J Am Chem Soc *133*, 1066-1076.

Meehan, S., Berry, Y., Luisi, B., Dobson, C.M., Carver, J.A., and MacPhee, C.E. (2004). Amyloid fibril formation by lens crystallin proteins and its implications for cataract formation. J Biol Chem *279*, 3413-3419.

Meier, B.H., and Earl, W.L. (1986). Excitation of Multiple Quantum Transitions under Magic Angle Spinning Conditions - Adamantane. J Chem Phys *85*, 4905-4911.

Meinhardt, J., Sachse, C., Hortschansky, P., Grigorieff, N., and Fandrich, M. (2009). Abeta(1-40) fibril polymorphism implies diverse interaction patterns in amyloid fibrils. J Mol Biol *386*, 869-877.

Mok, Y.F., Ryan, T.M., Yang, S., Hatters, D.M., Howlett, G.J., and Griffin, M.D. (2011). Sedimentation velocity analysis of amyloid oligomers and fibrils using fluorescence detection. Methods *54*, 67-75.

Monti, M., Amoresano, A., Giorgetti, S., Bellotti, V., and Pucci, P. (2005). Limited proteolysis in the investigation of beta2-microglobulin amyloidogenic and fibrillar states. Biochim Biophys Acta *1753*, 44-50.

Murakami, K., Hara, H., Masuda, Y., Ohigashi, H., and Irie, K. (2007). Distance measurement between Tyr10 and Met35 in amyloid beta by site-directed spin-labeling ESR spectroscopy: implications for the stronger neurotoxicity of Abeta42 than Abeta40. Chembiochem *8*, 2308-2314.

Myers, S.L., Thomson, N.H., Radford, S.E., and Ashcroft, A.E. (2006). Investigating the structural properties of amyloid-like fibrils formed in vitro from beta2-

microglobulin using limited proteolysis and electrospray ionisation mass spectrometry. Rapid Commun Mass Spectrom *20*, 1628-1636.

Naeem, A., and Amani, S. (2013). Deciphering structural intermediates and genotoxic fibrillar aggregates of albumins: a molecular mechanism underlying for degenerative diseases. PLoS One *8*, e54061.

Natalello, A., Frana, A.M., Relini, A., Apicella, A., Invernizzi, G., Casari, C., Gliozzi, A., Doglia, S.M., Tortora, P., and Regonesi, M.E. (2011). A major role for side-chain polyglutamine hydrogen bonding in irreversible ataxin-3 aggregation. PLoS One *6*, e18789.

Nath, S., Meuvis, J., Hendrix, J., Carl, S.A., and Engelborghs, Y. (2010). Early aggregation steps in alpha-synuclein as measured by FCS and FRET: evidence for a contagious conformational change. Biophys J *98*, 1302-1311.

Nayeem, S.M., and Deep, S. (2010). Rationalization of poor solubility of TGF-beta3 using MD simulation. Biochem Biophys Res Commun *401*, 544-547.

Nowak, M. (2004). Immunoglobulin kappa light chain and its amyloidogenic mutants: a molecular dynamics study. Proteins *55*, 11-21.

Oliveira, C.L., Behrens, M.A., Pedersen, J.S., Erlacher, K., and Otzen, D. (2009). A SAXS study of glucagon fibrillation. J Mol Biol *387*, 147-161.

Ono, K., Ikeda, T., Takasaki, J., and Yamada, M. (2011). Familial Parkinson disease mutations influence alpha-synuclein assembly. Neurobiol Dis *43*, 715-724.

Orte, A., Birkett, N.R., Clarke, R.W., Devlin, G.L., Dobson, C.M., and Klenerman, D. (2008). Direct characterization of amyloidogenic oligomers by single-molecule fluorescence. Proc Natl Acad Sci U S A *105*, 14424-14429.

Oztug Durer, Z.A., Cohlberg, J.A., Dinh, P., Padua, S., Ehrenclou, K., Downes, S., Tan, J.K., Nakano, Y., Bowman, C.J., Hoskins, J.L., *et al.* (2009). Loss of metal ions, disulfide reduction and mutations related to familial ALS promote formation of amyloid-like aggregates from superoxide dismutase. PLoS One *4*, e5004.

Palmieri, L.C., Melo-Ferreira, B., Braga, C.A., Fontes, G.N., Mattos, L.J., and Lima, L.M. (2013). Stepwise oligomerization of murine amylin and assembly of amyloid fibrils. Biophys Chem *180-181*, 135-144.

Paredes, J.M., Casares, S., Ruedas-Rama, M.J., Fernandez, E., Castello, F., Varela, L., and Orte, A. (2012). Early Amyloidogenic Oligomerization Studied through Fluorescence Lifetime Correlation Spectroscopy. Int J Mol Sci *13*, 9400-9418.

Petkova, A.T., Leapman, R.D., Guo, Z., Yau, W.M., Mattson, M.P., and Tycko, R. (2005). Self-propagating, molecular-level polymorphism in Alzheimer's beta-amyloid fibrils. Science *307*, 262-265.

Petkova, A.T., Yau, W.M., and Tycko, R. (2006). Experimental constraints on quaternary structure in Alzheimer's beta-amyloid fibrils. Biochemistry *45*, 498-512.

Pivato, M., De Franceschi, G., Tosatto, L., Frare, E., Kumar, D., Aioanei, D., Brucale, M., Tessari, I., Bisaglia, M., Samori, B., *et al.* (2012). Covalent alpha-synuclein dimers: chemico-physical and aggregation properties. PLoS One *7*, e50027.

Popova, L.A., Kodali, R., Wetzel, R., and Lednev, I.K. (2010). Structural variations in the cross-beta core of amyloid beta fibrils revealed by deep UV resonance Raman spectroscopy. J Am Chem Soc *132*, 6324-6328.

Pornsuwan, S., Giller, K., Riedel, D., Becker, S., Griesinger, C., and Bennati, M. (2013). Long-range distances in amyloid fibrils of alpha-synuclein from PELDOR spectroscopy. Angew Chem Int Ed Engl *52*, 10290-10294.

Provencher, S.W., and Glockner, J. (1981). Estimation of globular protein secondary structure from circular dichroism. Biochemistry *20*, 33-37.

Pryor, N.E., Moss, M.A., and Hestekin, C.N. (2012). Unraveling the Early Events of Amyloid-beta Protein (Abeta) Aggregation: Techniques for the Determination of Abeta Aggregate Size. Int J Mol Sci *13*, 3038-3072.

Rochet, J.C., and Lansbury, P.T., Jr. (2000). Amyloid fibrillogenesis: themes and variations. Curr Opin Struct Biol *10*, 60-68.

Rodrigues, J.R., Simoes, C.J., Silva, C.G., and Brito, R.M. (2010). Potentially amyloidogenic conformational intermediates populate the unfolding landscape of transthyretin: insights from molecular dynamics simulations. Protein Sci *19*, 202-219.

Saha, S., and Deep, S. (2013). Protein Aggregation: Elucidation of the Mechanism and Determination of Associated Thermodynamic and Kinetic Parameters. Current Physical Chemistry. In Press.

Saini, K., and Deep, S. (2010). Relationship between the wavelength maximum of a protein and the temperature dependence of its intrinsic tryptophan fluorescence intensity. Eur Biophys J *39*, 1445-1451.

Salvatore, D., Croguennec, T., Bouhallab, S., Forge, V., and Nicolai, T. (2011). Kinetics and structure during self-assembly of oppositely charged proteins in aqueous solution. Biomacromolecules *12*, 1920-1926.

Sarroukh, R., Goormaghtigh, E., Ruysschaert, J.M., and Raussens, V. (2013). ATR-FTIR: a "rejuvenated" tool to investigate amyloid proteins. Biochim Biophys Acta *1828*, 2328-2338.

Serpa, J.J., Patterson, A.P., Pan, J., Han, J., Wishart, D.S., Petrotchenko, E.V., and Borchers, C.H. (2013). Using multiple structural proteomics approaches for the characterization of prion proteins. J Proteomics *81*, 31-42.

Sharma, A., Agarwal, P.K., and Deep, S. (2010a). Characterization of different conformations of bovine serum albumin and their propensity to aggregate in the presence of N-cetyl-N,N,N-trimethyl ammonium bromide. J Colloid Interface Sci *343*, 454-462.

Sharma, A., Pasha, J.M., and Deep, S. (2010b). Effect of the sugar and polyol additives on the aggregation kinetics

of BSA in the presence of N-cetyl-N,N,N-trimethyl ammonium bromide. J Colloid Interface Sci *350*, 240-248.

Shashilov, V., Xu, M., Ermolenkov, V.V., Fredriksen, L., and Lednev, I.K. (2007). Probing a fibrillation nucleus directly by deep ultraviolet Raman spectroscopy. J Am Chem Soc *129*, 6972-6973.

Shivu, B., Seshadri, S., Li, J., Oberg, K.A., Uversky, V.N., and Fink, A.L. (2013). Distinct beta-Sheet Structure in Protein Aggregates Determined by ATR-FTIR Spectroscopy. Biochemistry. In Press.

Silva, R.A., Barber-Armstrong, W., and Decatur, S.M. (2003). The organization and assembly of a beta-sheet formed by a prion peptide in solution: an isotope-edited FTIR study. J Am Chem Soc *125*, 13674-13675.

Singh, P.K., Kotia, V., Ghosh, D., Mohite, G.M., Kumar, A., and Maji, S.K. (2013). Curcumin modulates alpha-synuclein aggregation and toxicity. Acs Chem Neurosci *4*, 393-407.

Smirnova, E., Chebotareva, N., and Gurvits, B. (2013). Transient transformation of oligomeric structure of alpha-crystallin during its chaperone action. Int J Biol Macromol *55*, 62-68.

Smith, D.P., Radford, S.E., and Ashcroft, A.E. (2010). Elongated oligomers in beta2-microglobulin amyloid assembly revealed by ion mobility spectrometry-mass spectrometry. Proc Natl Acad Sci U S A *107*, 6794-6798.

Sreerama, N., and Woody, R.W. (1993). A self-consistent method for the analysis of protein secondary structure from circular dichroism. Anal Biochem *209*, 32-44.

Tashiro, M., Kojima, M., Kihara, H., Kasai, K., Kamiyoshihara, T., Ueda, K., and Shimotakahara, S. (2008). Characterization of fibrillation process of alpha-synuclein at the initial stage. Biochem Biophys Res Commun *369*, 910-914.

Torok, M., Milton, S., Kayed, R., Wu, P., McIntire, T., Glabe, C.G., and Langen, R. (2002). Structural and dynamic features of Alzheimer's Abeta peptide in amyloid fibrils studied by site-directed spin labeling. J Biol Chem *277*, 40810-40815.

Trexler, A.J., and Rhoades, E. (2013). Function and dysfunction of alpha-synuclein: probing conformational changes and aggregation by single molecule fluorescence. Mol Neurobiol *47*, 622-631.

Tycko, R. (2006). Molecular structure of amyloid fibrils: insights from solid-state NMR. Q Rev Biophys *39*, 1-55.

Tycko, R. (2011). Solid-state NMR studies of amyloid fibril structure. Annu Rev Phys Chem *62*, 279-299.

Uversky, V.N., and Fink, A.L. (2004). Conformational constraints for amyloid fibrillation: the importance of being unfolded. Biochim Biophys Acta *1698*, 131-153.

Vestergaard, B., Groenning, M., Roessle, M., Kastrup, J.S., van de Weert, M., Flink, J.M., Frokjaer, S., Gajhede, M., and Svergun, D.I. (2007). A helical structural nucleus is the primary elongating unit of insulin amyloid fibrils. PLoS Biol *5*, e134.

Wang, X., Kumar, S., Buck, P.M., and Singh, S.K. (2013). Impact of deglycosylation and thermal stress on conformational stability of a full length murine IgG2a monoclonal antibody: observations from molecular dynamics simulations. Proteins *81*, 443-460.

Williams, D.M., and Pukala, T.L. (2013). Novel insights into protein misfolding diseases revealed by ion mobility-mass spectrometry. Mass Spectrom Rev *32*, 169-187.

Wishart, D.S., Sykes, B.D., and Richards, F.M. (1991). Relationship between nuclear magnetic resonance chemical shift and protein secondary structure. J Mol Biol *222*, 311-333.

Xu, M., Ermolenkov, V.V., Uversky, V.N., and Lednev, I.K. (2008). Hen egg white lysozyme fibrillation: a deep-UV resonance Raman spectroscopic study. J Biophotonics *1*, 215-229.

Xu, M., Shashilov, V., and Lednev, I.K. (2007). Probing the cross-beta core structure of amyloid fibrils by hydrogen-deuterium exchange deep ultraviolet resonance Raman spectroscopy. J Am Chem Soc *129*, 11002-11003.

Yamamoto, S. (2012). Conformational analyses of peptides and proteins by vibrational Raman optical activity. Anal Bioanal Chem *403*, 2203-2212.

Yamamoto, S., and Watarai, H. (2012). Raman optical activity study on insulin amyloid- and prefibril intermediate. Chirality *24*, 97-103.

Yanagi, K., Sakurai, K., Yoshimura, Y., Konuma, T., Lee, Y.H., Sugase, K., Ikegami, T., Naiki, H., and Goto, Y. (2012). The monomer-seed interaction mechanism in the formation of the beta2-microglobulin amyloid fibril clarified by solution NMR techniques. J Mol Biol *422*, 390-402.

Yoshimura, Y., Lin, Y., Yagi, H., Lee, Y.H., Kitayama, H., Sakurai, K., So, M., Ogi, H., Naiki, H., and Goto, Y. (2012). Distinguishing crystal-like amyloid fibrils and glass-like amorphous aggregates from their kinetics of formation. Proc Natl Acad Sci U S A *109*, 14446-14451.

Yoshimura, Y., Sakurai, K., Lee, Y.H., Ikegami, T., Chatani, E., Naiki, H., and Goto, Y. (2010). Direct observation of minimum-sized amyloid fibrils using solution NMR spectroscopy. Protein Sci *19*, 2347-2355.