Analysis of Protein Aggregation in Neurodegenerative Disease

Pathological protein and peptide aggregation are key events in a number of chronic and devastating neurodegenerative conditions including dementias such as Alzheimer’s and Creutzfeldt-Jakob’s disease and other central nervous system diseases such as Parkinson’s and Huntington’s disease and amyotrophic lateral sclerosis. Analytical methods for studying protein aggregation in these diseases are important for mapping pathophysiological events and ultimately for the development of new therapies and better diagnostic tools.

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Many neurodegenerative diseases including Alzheimer’s (AD), Parkinson’s (PD), and Huntington’s disease (HD) are associated with the occurrence of disease-specific misfolded and aggregated proteins and peptides in the damaged neurological tissues.1,2 Overall, more than half of the 25 recognized neurodegenerative diseases are associated with specific protein aggregates. The group also includes amyotrophic lateral sclerosis (ALS), Huntington’s disease and other polyglutamine diseases (at least 8 additional distinct entities), the serpinopathies, and the prion diseases (Table 1). Neurodegenerative diseases are prevalent and the number of cases is increasing. AD, for example, affects 1% of people at the age of 60 and the incidence then doubles every fifth year so that 30–50% of people >85 years are affected. Because of the predicted increase in life expectancy this means that the absolute numbers of AD victims in North America are expected to reach 6 million by 2050 from around 2 million today. Causes and effects linking protein aggregation and the degenerative central nervous system (CNS) diseases are largely unknown but the aggregating proteins are diagnostically specific3 and the association of disease with aggregation of specific proteins and specific forms of aggregation has obvious diagnostic and therapeutic implications. Thus, for the amyloid type of fibrillar aggregates in vivo imaging may be accomplished using specific imaging molecules for amyloid-β (Aβ)4,5 or general imaging of systemic amyloids.6,7 In addition, treatment based on small molecule inhibitors is being developed and applied to inhibit amyloid fibrillation.8,9

Most methods for direct analysis of protein–protein interactions require that the proteins are in solution. This is true for electrophoretic, mass spectrometric, chromatographic, centrifugal, and many spectroscopic methods. The protein aggregates associated with the neurological diseases in focus here, however—especially those that adopt an amyloid configuration—are extremely insoluble and thus pose considerable challenges for direct analysis. Also, X-ray crystallography and other methods that require protein crystallization are not useful for characterizing heterogeneously sized oligomers, polymers, and amorphous aggregates or insoluble amyloids of proteins. Thus, in many cases much is known about conformational states and interactions of native or near-native, still soluble proteins, i.e., the very early stages of protein misfolding and oligomerization. At the other extreme of the molecular pathology pathway much is known about the generic amyloid fold and the identity of the aggregating proteins in the various diseases. The persistent analytical challenge is the characterization of the intermediate folding and oligomerization dynamics that connect these two extremes. By achieving this goal it will hopefully be possible to generate new approaches for treatment. This article reviews key techniques, established and evolving, for analysis of protein aggregation, early, intermediate, and late stages, ordered or unordered, with emphasis on examples from the analysis of proteins involved in neurodegenerative disease.

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## TYPES OF PROTEIN AGGREGATION IN NEURODEGENERATION

Protein function and solubility often require that the protein attains one or more specific conformations, but not all proteins have specific folding patterns. Some are intrinsically disordered (also called natively unfolded)—e.g., the microtubule tau protein and possibly the α-synuclein (αSN) found in Lewy bodies in PD and other synucleinopathies. Additionally, some proteins may contain domains of unordered structure, exist in conformational equilibria, or exhibit domains with conformational fluctuations or short-lived transitions to unfolded intermediates, while others are much more structurally well-defined and conformationally constrained. Thus, the ordered fibrillar aggregation typical of amyloid-forming proteins depends on a regular, stacked β-sheet structure which may be generated through partially unfolded intermediates. In contrast,

### Table 1

<table>
<thead>
<tr>
<th>Disease</th>
<th>Aggregating Protein/Peptide</th>
<th>Aggregate Structure</th>
<th>Comments</th>
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</thead>
<tbody>
<tr>
<td>Alzheimer’s Disease (AD)</td>
<td>Amyloid β-peptide 1−40/1−42</td>
<td>Amyloid</td>
<td>Peptide isoforms</td>
</tr>
<tr>
<td>Parkinson’s Disease (PD)</td>
<td>Tau protein</td>
<td>Amorphous/some amyloid features</td>
<td>Hyperphosphorylated isoforms</td>
</tr>
<tr>
<td>Multisystem Atrophy</td>
<td>α-synuclein</td>
<td>Amorphous</td>
<td>Glial cell cytoplasmic inclusion bodies</td>
</tr>
<tr>
<td>Frontotemporal Dementia (FTLD-tau)</td>
<td>Tau protein</td>
<td>Amorphous/some amyloid features</td>
<td>Hyperphosphorylated isoforms</td>
</tr>
<tr>
<td>Amyotrophic Lateral Sclerosis (ALS)</td>
<td>Neurofilament protein(?), SOD1 in familial ALS, DNA/RNA binding proteins (FUS) in subsets of ALS/FTLD, colocalizes with TDP-43 and ubiquitin</td>
<td>Disordered aggregates in neurons and astrocytes and other glial cells, Subsets with amyloid features</td>
<td>Allen inclusions bodies</td>
</tr>
<tr>
<td>Huntington’s Disease (HD)</td>
<td>Huntingtin</td>
<td>Nuclear inclusion bodies</td>
<td>Huntingtin with polyglutamine extension</td>
</tr>
<tr>
<td>Other Polyglutamine diseases (8 known in addition to HD)</td>
<td>Ataxins, atrophin-1, androgen receptor</td>
<td>Nuclear inclusion bodies</td>
<td>Proteins with polyglutamine extension</td>
</tr>
<tr>
<td>Prion Diseases</td>
<td>Prion protein</td>
<td>Amyloid</td>
<td>Prion protein mutations or wild-type</td>
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<td>Familial amyloidotic polyecephalopathy</td>
<td>Transthyretin, apolipoprotein A1, gelolin</td>
<td>Amyloid</td>
<td>Transthyretin mutations or wild-type</td>
</tr>
<tr>
<td>Serinopathies (familial encephalopathy)</td>
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</tr>
<tr>
<td>Neurodegenerative disorders and normal aging</td>
<td>Actin and other</td>
<td>Cytoplasmic inclusion (Hirano) bodies</td>
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</tr>
</tbody>
</table>

### Figure 1.
Schematics of stages of amorphous and amyloid protein aggregation with relevant methods for investigation at each stage. The mechanism of aggregate formation (both amyloid and amorphous) is depicted as a series of consecutive reaction steps beginning with the native monomer (I) unfolding to a partially folded intermediate (II). Unfavorable self-assembly of unfolded monomers results in a nucleus (III). Hereafter, oligomers are formed through favorable monomer addition (IV), and eventually mature fibrils (Va) or amorphous aggregates (Vb) are formed. Monomer loss and oligomer/aggregate growth time profiles are shown for each step. Note that the monomer and oligomer concentration will not completely reach zero. However, the concentration may decrease below the detection limit of the instrument. The Lag, elongation, and plateau phases relate to the formation of aggregates. The soluble oligomers and fibrils are depicted as single species for simplicity. Asterisks mark techniques that are described in this paper. The type of information that is attainable by each technique is indicated by superscripts at first entry in the figure: C, conformation; D, dynamics; K, kinetics; S, structure/size.
protein aggregates of no specific ultrastructure simply may arise as a consequence of increases in protein concentration and/or specific post-translational modifications related to a disease state. For many proteins in neurodegenerative diseases, the end-stage aggregated structure, however, is the fibrillar amyloid deposit. These deposits are characterized by positive staining with Congo red (birefringent) or thioflavin T and by the signature parallel β-sheet structure differentiation patterns observed by X-ray fiber diffraction analysis.

Not all protein aggregates in CNS diseases are of the amyloid fibrillar type. Thus, in AD the tau protein forms nonstructured aggregates intracellularly in neuronal tissue (although some authors classify this as intracellular amyloid12). The αSN forms unstructured aggregates called Lewy bodies intracellularly in the cytoplasm of neurons in PD. Also, the same species, such as the Aβ peptides in AD and the TDP-43 protein in amyotrophic lateral sclerosis13 may coexist as amorphous aggregates and amyloid fibrils partially dependent on the anatomical site of aggregation. In contrast, it has been demonstrated that serpins in the serpinopathies retain their native structure in the polymer that is generated by β-sheet expansion after domain swapping.14

Soluble oligomeric precursors to neuropathological amyloid are believed to be the actual toxic species that damages tissues and it is helpful to divide the methods for analysis of protein aggregation in two groups, i.e., methods for analysis in solution and methods for solid state analysis. The methods may also be divided into hydrodynamic methods where external fields (forces) are applied, methods where external probes (such as antibodies or dyes) are used, and optical methods (e.g., spectroscopy) that detect intrinsic characteristics of the molecules such as fluorescence in the absence of applied force fields or external probes. The most useful methods combine one or more elements from more than one group, e.g., separation step(s) combined with information-rich detection methods. Whichever approach is chosen, the key challenge lies in characterizing the steps between the soluble native monomers and the insoluble aggregated states, i.e., the conformational change(s) and self-assembly processes that precede protein aggregation (Figure 1). It is important to realize that protein aggregation is a highly complex process where a single technique cannot give a complete characterization of the entire chain of events. Each step of the protein aggregation process is also by itself very heterogeneous and may give rise to oligomeric species with a distribution of size.
and conformational properties which are not readily resolved and quantitatively estimated using any of the present techniques. Thus, the applicability of specific analytical techniques varies along the protein aggregation pathway. Depending on which aspect of protein aggregation is going to be probed, different techniques are preferred (Figure 1). Experiments will often be limited by factors such as sample availability, purity, and concentration attainable. We here focus on a broad spectrum of techniques ranging from separation techniques to immunoassays, and although the list is not exhaustive it covers many of the most commonly used analytical techniques for studying protein aggregation.

■ SEPARATIVE METHODS: ELECTROPHORESIS

In electrophoresis (the transport of charged molecules in the presence of an electrical field) (Figure 2a) size, shape, and charge are the main molecular parameters determining selectivity. This means that electrophoresis is well suited for characterizing protein oligomers provided that they are soluble and stable to the separation conditions and that they are analyzed in pure form or are detectable when present in complex mixtures such as biofluids. As an example, polyacrylamide gel electrophoresis (PAGE) readily separates Aβ monomers and oligomers after covalent cross-linking.15 The use of SDS-PAGE for oligomer characterization is not straightforward since the anionic detergent SDS in some studies (e.g., with Aβ peptides) has been shown to induce oligomerization.16 Kinetics analysis of the aggregation process using capillary electrophoresis (CE) may be based on monitoring changes in the concentration of both monomers and early, still soluble oligomers over time as demonstrated for both Aβ17 and insulin oligomers.18 Alternatively, CE can be coupled with specialized detectors, e.g., laser-induced fluorescence anisotropy (LIFA) detectors for online assessment of the size/shape of the separated Aβ species.19 In this case, information relating to size and shape of the analytes may be obtained but electrophoretic methods are generally not able to provide much structural information about the separated analytes. CE is well suited for monitoring the contents of small biological samples, including microdialysis fluids, over time, due to its very low sample volume consumption (a few small biological samples, including microdialysis analytes. CE is well suited for monitoring the contents of small biological samples, including microdialysis fluids, over time, due to its very low sample volume consumption (a few microliters is typically injected in each analysis and thus a 20 μL sample volume is enough for many hundred analyses). Also, in affinity-CE mode the technique is easily combined with molecular probes of amyloid-like structure such as thioflavin T.19 Congo red, or probes of hydrophobic environments such as 1-anilino-8-naphthalene-sulfonate (ANS).20–22

In native gel electrophoresis (no anionic detergent present), aggregates are more likely to be stable than in denaturing electrophoresis and this approach has been used, e.g., to analyze Aβ and yeast prion aggregates23 and to analyze oligomer formation of neuroserpin variants.24 Native gel electrophoresis, however, is not well suited for exact molecular weight estimates because separation in the absence of anionic detergent is dependent on a combination of analyte charge, shape, and mass.25 For strongly complexed aggregates denaturing gel electrophoresis may be useful. As an example, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) is widely used to characterize soluble Aβ oligomers and prion protein aggregates that are SDS-resistant.26,27,23 The αSN nonamyloidogenic aggregates formed in the presence of dopamine (giving rise to covalent intermolecular links) were also analyzed in this way28 and found by spectroscopic and X-ray fiber diffraction methods to be lacking well-defined secondary structure. SDS-PAGE is also conveniently used to study aggregate size by chemical cross-linking samples prior to electrophoresis.29 In studies of polyglutamine aggregates of the MJJD protein SDS-PAGE was used to show a dependency of aggregate stability on chronological age in a Drosophila model of human Machado-Joseph disease (spinocerebellar ataxia type 3, SCA3). In this way an age-dependent association between decreasing aggregate stability and increasing neurotoxicity was demonstrated.30 The main drawback of native electrophoresis is that it cannot separate oligomers with an identical size-to-charge ratio since this gives rise to identical electrophoretic mobility and hence migration time, and for non-SDS resistant oligomers a denaturing (SDS-PAGE) electrophoresis will not be applicable because oligomers are dissociated. Also, like all separations techniques electrophoresis is an ex situ method, meaning that the equilibrium of the aggregating species is disturbed during separation.

■ SEPARATIVE METHODS: SIZE-EXCLUSION CHROMATOGRAPHY (SEC)

Size exclusion chromatography (SEC) is a liquid flow-driven separation technique where the hydrodynamic volume (i.e., analyte size and shape) determines analyte path length and thus the time to emerge at the detector (Figure 2b). Smaller hydrodynamic volumes equal longer pathlengths because small analytes have a larger mobile phase distribution volume in the separation column which contains beads with pores that smaller molecules can enter. Coupled with refractive index and multiangle static light scattering detectors aggregation numbers can be estimated at the same time as the relative amounts of different oligomers.31,32 Separation times and protein concentration requirements restrict the applicability of SEC to situations with nonlimiting amounts of material and to situations where the nonequilibrium separation conditions are not interfering with the interpretation of results. The quite stable Aβ early oligomers have been analyzed by SEC in several studies.33,34 Analytical SEC separates Aβ monomers from dimers and higher order oligomers and is useful for the preparative isolation of well-defined monomers or oligomers for further studies.26,29 SEC has been extensively used—with conflicting results—for characterizing αSN from various sources.35–38 Even though SEC columns are designed for different molecular size ranges, the approach will normally be useful in the case of higher order oligomers, polymers, and fibrillar species which will either not enter the bed of the column at all or elute with the void volume without size separation.

■ SEPARATIVE METHODS: ANALYTICAL ULTRACENTRIFUGATION

One of the important alternatives to SEC is sedimentation velocity and sedimentation equilibrium analytical ultracentrifugation (AUC) (Figure 3). AUC is a classical hydrodynamic technique for analysis of macromolecular properties.39 Sedimentation velocity experiments measure the velocity of an analyte subjected to a radial acceleration of several hundred thousand g (Figure 3b). Separation is solely dependent on sedimentation coefficients (the steady-state velocity per unit centrifugal field) which correlate directly with molecular mass and inversely with the frictional coefficient (that equals molecular size). The sedimentation equilibrium methods use
is under ideal conditions an exponential function of the radial distance achieved after a su-
stance is present in the sample cell is determined. In sedimentation equilibrium analysis (c), the centrifugal
force is much smaller than in a sedimentation velocity experiment, and an equilibrium distribution of the molecules in the sample cell is achieved after a sufficiently long time. The concentration distribution is under ideal conditions an exponential function of the radial distance and depends on the molecular weight and the radial acceleration.

much lower rotor speeds and output is the equilibrium distribution of the analyte (Figure 3c). The shape of this distribution is proportional to molecular weight and is independent of friction. AUC is very well suited for assessing the distribution of aggregate sizes since an experiment covers a size range in molar mass of 3 orders of magnitude. Thus, the sedimentation velocity AUC approach is very well suited for characterizing the molecular weight distribution of proteins in relevant solutions but mostly in situations where the quantity of biological materials, e.g., as in the quality control of biopharmaceuticals, is not a limiting factor. Moreover, AUC may be used for evaluating noncovalent interactions in a 2 (maximum 3) component protein solution under equilibrium conditions provided enough material is available at a suitable concentration and in not too complex mixtures (a few hundred micrograms at 95% purity). Sedimentation equilibrium analytical ultracentrifugation was used to provide data suggesting tetramer assembly of native αSN which contrasts with the prevailing view of this PD-related aggregating protein normally being an unstructured monomer. 

A drawback of AUC is that it is time-consuming; thus, equilibrium centrifugation experiments may require several days. However, in contrast to electrophoretic and SEC techniques, the centrifugation approaches do not need molecular weight standards for calibration and provide direct measurements of the molecular weights of analytes in their native state in solutions without assumptions about their conformation and shape.

Figure 3. Principles of analytical ultracentrifugation. (a) Schematic illustration of an analytical ultracentrifuge, and the two most important modes, (b) sedimentation velocity analysis and (c) sedimentation equilibrium analysis. Separation of the analytes is achieved by applying a large radial acceleration (>100 000g in sedimentation velocity analysis), causing the particles to move away from the center with a sedimentation velocity v_s. The sample cell for a sedimentation analysis experiment is shown. In the sedimentation velocity analysis (b), the rate and shape of the moving sedimentation boundary are monitored by scanning the sample at different time points, which allows the sedimentation coefficient and translational diffusion coefficient to be determined. In sedimentation equilibrium analysis (c), the centrifugal force is much smaller than in a sedimentation velocity experiment, and an equilibrium distribution of the molecules in the sample cell is achieved after a sufficiently long time. The concentration distribution is under ideal conditions an exponential function of the radial distance and depends on the molecular weight and the radial acceleration.

SEPARATIVE METHODS: MASS SPECTROMETRY AND ION MOBILITY MASS SPECTROMETRY

In mass spectrometry (MS) molecules are separated as molecular ions in the gas phase based on their mass-to-charge (m/z) ratios (Figure 4a). The samples are either ionized and volatilized by matrix-assisted laser desorption ionization (MALDI) of liquid samples from direct injection or from an online separation system. Data output is intensity as a function of m/z values (the mass spectrum). MS is extensively used to characterize and monitor the formation of ionizable non-covalent oligomers and aggregates in vitro and yields precise data on aggregate stoichiometry and mass distribution. In addition, MS can be used for mapping of post-translational modifications, and for semiquantitative characterization of proteins in complex biological fluids. MS has a very high selectivity and requires little material (down to a few microliters at nanomolar concentration). Laser desorption or surface layer ejection by clustered ion beams (the latter most useful for molecules <2 000 molecular mass) may also be performed directly on tissues (tissue imaging MS or tissue imaging secondary ion MS) including neuropathological samples containing Aβ deposits. Albeit still under development, this provides a direct, antibody-independent identification of the origin of proteins in aggregates ex vivo including potentially their post-translational modifications at specific residues.

ESI and desorption-ESI are mild ionization techniques that allow the analysis of very large ionizable molecular complexes. However, the analysis requires molecules in solution which may pose a problem since amyloidogenic proteins aggregate and precipitate out of solution over time. By MALDI-MS it is possible to study insoluble species (Figure 4a), but this approach is not readily compatible with online dynamic methods for studying the evolution of folding intermediates. The process of drying down a sample in a mixture with a matrix solution of organic acids also is prone to disrupt noncovalent complexes. Finally, MALDI typically yields monovalent ions resulting in broad, imprecisely defined mass peaks for large proteins whereas the multivalent ions generated by ESI result in much more precise mass assignments. In ESI the analysis of charge state distribution enables the estimation of the population of various conformational states in a given protein as has been shown for monomeric αSN conformers.

Recently, MS was combined with a preceding gas-phase electrophoretic separation step in a combined approach called gas-phase electrophoretic molecular analysis or ion mobility spectrometry-MS (IMS-MS) (Figure 4b). Here, ESI-generated ions are charge-reduced and separated according to their size-dependent electrophoretic mobility in air or a gas such as helium. This allows the virtues of MS to be expanded by utilizing a two-dimensional separation approach where the ion mobility separation in the first dimension allows separation of complex ionic mixtures according to molecular size (cross-sectional shape) and charge. The first dimensional drift tube contains a buffer gas that leads to separation of ions based on their collisional cross-section in a process similar to plasma chromatography. The second dimension is the time-of-flight MS analysis that may be coupled to the first dimension in different ways and links every charge state with its collisional cross section. Thus, conformers with the same masses but different size (e.g., different conformations of the same species)
Figure 4. Mass spectrometry: (a) The principle of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is shown. The MS matrix is ionized and vaporized by a laser pulse. Charges from the ionized matrix are transferred to the sample molecules which are then accelerated by applying an electric field in the TOF mass spectrometer. To improve resolution the accelerated ions are slowed down and turned left by a reflector. For ions with the same kinetic energy, the lighter ions reach the detector first. The experimental data (upper right) shows separation of Aβ(1–40) multimers (from tetramers and up). (b) Ion mobility spectrometry-MS (IMS-MS) adds a first dimension separation of ions according to their molecular collisional cross-sectional size when traveling through a gas-filled drift cell (ion mobility separation) to the subsequent second dimension TOF-MS separation. The figure shows separation of a single charged monomer and double charged dimer that have equal m/z but different size and charge. The double-charged dimer travels faster through the ion mobility drift cell, and hence separation is achieved in the first dimension. For two distinct conformations of, e.g., a single charged monomer, the most compact conformation will travel faster in the ion mobility drift cell (not shown).

Figure 5. Spectrometry methods: (a) Turbidity, oligomers and aggregates with a hydrodynamic radius larger than the wavelength of the incident lights scatter the light. This causes a decrease in transmitted light and hence an increase in absorption. For homogeneous samples, the total turbidity is proportional to the amount of formed oligomers and hence the progression of the aggregation process can be monitored. (b) Dynamic light scattering, oligomers and aggregates present in solution cause fluctuations of the incident light, which are related to the hydrodynamic radius (R_H) of the molecules. The increase in R_H with time can be followed to give information on fibril elongation rates (bottom left curve). Alternatively, the relative size distribution of the oligomers may be determined (bottom right curve). (c) ThT fluorescence spectroscopy, the fluorescent dye ThT exhibits a marked increase in fluorescence upon binding to amyloid fibrils with an excitation maximum at around 450 nm and an emission maximum at around 480 nm (bottom right curve). Progression of amyloid formation can be monitored by following the increase in fluorescence with time which often results in the characteristic sigmoid fibril growth curves (bottom left). The fluorescence intensity may vary between fibrils depending on underlying structure and morphology (bottom right).
will be separated—usually with the lowest charge states as the smallest ions. Also, different oligomeric species with identical $m/z$ value but different absolute size and charge may be separated (Figure 4b). ESI-IMS-MS is very useful for probing very early unfolding events on the path to aggregation. Also, the method is promising for the characterization of oligomeric intermediates. This was shown in studies of the role of conformers and truncated species in the oligomerization and aggregation of $\alpha$SN and $\beta$-amyloid and in studies of $\beta$-sheet formation from oligomeric globular conformations by small peptides, including a peptide from the amyloidogenic yeast prion protein.59 ESI-IMS-MS is also useful for studies of small molecule interactions with $\beta\beta$ isoforms.60,61

Hydrogen–deuterium (H/D) exchange monitored by MS is used to measure the dynamics of conformational fluctuations in proteins. The approach exploits the fact that the hydrogens that are protected by structure, e.g., involved in hydrogen bonding, exchange much more slowly than the accessible free hydrogens of backbone amides. When freed from hydrogen bonding by a structural change, the previously inaccessible amide hydrogens are exposed to solvent and readily exchange with deuterium in the solvent. The measurements thereby differentiate between states with or without protecting structures. The approach has been used to investigate the unstructured/structured core state of soluble and fibrillar $\alpha$SN62–64 and has shown that fibrils are more dynamic structures than previously assumed.64,65 H/D exchange can also be conveniently monitored by NMR spectroscopy (see below) since the nuclei of hydrogen and deuterium have different magnetic properties.

■ SPECTROSCOPY: TURBIDITY AND LIGHT SCATTERING

Turbidity is an optical kinetic method that detects the formation of oligomers and aggregates with a hydrodynamic radius $R_H$ greater than the wavelength of the incident light, $\lambda_i$.66,67 A decrease in the transmitted light, i.e., an increase in absorbance/turbidity, is observed when oligomers and aggregates that are large enough to scatter light are present in the solution (Figure 5a). If the oligomers are homogeneous with respect to size and shape, the total turbidity is proportional to the amount of oligomers with $R_H$ larger than $\lambda_i$.68 Usually a $\lambda_i$ around 350 nm is used since soluble monomeric proteins usually do not absorb at this wavelength. The aggregation assay is simple and may be performed in a multiwell plate reader format thereby allowing high throughput. Also, the fact that the protein does not need to be labeled is an advantage. The main disadvantage of turbidity measurements is that there is no discrimination between different types of aggregates (e.g., amyloid versus nonamyloid), a low detection sensitivity, and no ability to detect oligomeric intermediates with a $R_H$ smaller than $\lambda_i$. Also, morphological and structural changes of the aggregates may be missed, and large differences in aggregation data between turbidity and other methods such as fluorescent dyes and electron microscopy have been observed for $\beta\beta$ and $\alpha$SN.70 Thus, complementary independent techniques are needed to support turbidity data, especially when the aim is to follow quantitatively the effects of factors such as pH, ionic strength, or ligands on the aggregation events.

Dynamic light scattering (DLS), also referred to as quasielastic light scattering (QELS), is an in situ, sensitive, and label-free method to determine the size distribution of formed oligomers and aggregates as well as aggregation kinetics. The size range of $R_H$ that can be detected is very wide spanning from around 1 nm up to 4 $\mu$m.71 Since the molecular weight is proportional to the cube of $R_H$, the molecular weight range of the molecules that can be detected spans almost 11 orders of magnitude. In DLS the sample is irradiated by a light source, and the fluctuations of the scattered light intensity is detected (Figure 5b). This fluctuation is caused by the Brownian motions of the molecules and is a measure of the molecular diffusion coefficient. Small molecules diffuse faster than larger molecules so the small molecules will cause less fluctuation in the intensity of the scattered light. The diffusion coefficient can be determined from the scattering fluctuation by computing the autocorrelation function.72 The diffusion coefficient is dependent on the size and shape of the molecule. The relation between $R_H$ and the diffusion coefficient is given by the Stokes–Einstein equation for spherical molecules. Since many of the proteins in neurodegenerative diseases form nonspherical amyloid fibrils, an apparent $R_H$ can be calculated.73 If the range of $R_H$ in the system is relatively narrow it may be convenient to calculate an average hydrodynamic radius.74 However, it may be more useful to obtain a size distribution of the oligomers by fitting the autocorrelation function to mathematical models (Figure 5b). This may give important information on how different oligomeric species evolve with time as shown for $\beta\beta$.75 DLS is only qualitative when determining size-distributions. However, it gives quantitative data for $R_H$, so if the increase in $R_H$ with time is followed the data may be used to determine elongation rates and fibril lengths.74 Thus, the method has been used to follow the aggregation kinetics of $\beta\beta$, $\alpha$SN,74 and huntingtin.4,76,77

Since DLS, like turbidity, is not selective for any type of particle, the method is very sensitive to large impurities such as dust particles. The bias in sensitivity toward large impurities stems from the fact that the intensity of scattering by a particle is proportional to the square of its mass.72 This means that an oligomer consisting of 100 monomers (or a dust particle with the same mass) scatters as much as 10 000 (1002) monomers separately. Hence, filtration or centrifugation of the sample may be required prior to detection to remove impurities or preformed oligomers and aggregates. Fibril–fibril interactions also may lead to erroneous interpretations when analyzing DLS data of amyloidogenic proteins. Fibril–fibril interactions slow down diffusion and thereby lead to overestimation of fibril length.72 Finally, data analysis and interpretation can be quite complex when the sample is polydisperse, and determining the size distribution by fitting the experimentally measured autocorrelation function is mathematically challenging.73

■ DYES AND EXTRINSIC FLUORESCENT PROBES

Staining with the dye Congo Red (CR) has been used for histological detection of amyloid fibrils for almost 100 years and is considered a defining criterion of amyloid.78 When CR is bound to amyloid fibrils, it exhibits apple-green birefringence under polarized light.79 It is primarily used in diagnosis of ex situ tissue. The use of CR for in situ monitoring of protein aggregation is not straightforward since CR may interfere with the aggregation of various different amyloid proteins (see, e.g., refs 80 and 81). Hence, CR mimics have been investigated as amyloidosis inhibitors80 including studies in animal models to investigate ameliorating effects of CR in different neurodegenerative diseases such as AD, HD, and prion diseases (reviewed in ref 78). The exact binding mechanism between CR and amyloid fibrils is not well understood and may in fact differ between different proteins.79

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The fluorescent dye thioflavin T (ThT) is widely used for probing the presence of amyloid fibrils and especially for in situ monitoring of amyloid formation kinetics, since it generally does not affect the aggregation kinetics. ThT exhibits a marked increase in fluorescence upon binding to amyloid fibrils with an excitation maximum at around 450 nm and an emission maximum at around 480 nm. The formation of amyloid fibrils can be probed by monitoring the increase in fluorescence as a function of time (Figure 5c). As is the case with turbidity, the ThT assay can be performed in a multiwell plate reader format. Although often assumed, the ThT fluorescence intensity is not an absolute measure of the amount of formed amyloid (and indeed not completely specific for amyloid folds) and a saturation effect may be observed at high protein concentrations. Hence, linearity between fluorescence intensity and protein concentration should be verified by a standard curve if the fluorescence intensity at the plateau phase is used quantitatively, e.g., to quantify the total amount of aggregated material. Importantly, the ThT fluorescence may be altered dramatically by interactions of the protein with ligands that can modify aggregate structure and morphology such as metal ions and β-sheet antagonists. Thus, when the ThT assay is used for probing the effects of such ligands, it should be complemented with techniques that probe the ultrastructure of the aggregates.

## ULTRASTRUCTURAL IMAGING METHODS

While light microscopy of CR-stained tissue sections readily reveals amyloid deposits (cf. above), visualization of the morphology of insoluble protein deposits requires high-resolution (subnanometer scale) ultrastructural methods such as electron microscopy (EM) and atomic force microscopy (AFM) (Figure 6). EM was used for the first ever imaging of amyloid deposits that showed the presence of the characteristic structural core elements, i.e., long, unbranched fibrils. EM methods are useful for examining the morphology of aggregates especially in the case of ordered aggregates such as prefibrillar and fibrillar species. Classical EM (transmission EM) uses a high-voltage focused electron beam that passes through the specimen in vacuo and forms an image that depends on the scattering of the electrons by the specimen. In most instances the imaging is enhanced by negative staining (heavy metals such as uranyl acetate) or rotary shadowing (application of heavy metals by evaporation to coat specimens). EM methods may be combined with antibodies labeled with gold particles to pinpoint specific epitopes, e.g., coaggregated proteins or the epitopes of the aggregating protein itself (see below). TDP-43 aggregates seen in subsets of ALS cases were characterized in this way. The resolution of negative staining EM is rather limited (about 25 Å). Also, the staining protocols including nonphysiological pH and ionic strength and the dehydration, high vacuum, and the intense radiation involved in EM are prone to generate artifacts in sensitive biological samples. In such cases cryo-EM which allows the examination of unstained and unfixed specimens may be applied. This technique entails working with unstained, rapidly frozen hydrated, native specimens preserved in vitrified (noncrystalline) water at −180 °C using low-to-moderate radiation dose EM. Even though extensive image noise reduction must be applied because of the inherent low contrast, cryo-EM imaging has the potential of near atomic resolution. Cryo-EM has been used to characterize amyloid fibrils of Aβ, αSN, yeast prion, and the protein polymers of the serpinopathies. By computationally combining tilted EM images of cryosectioned vitrified specimens, 3D-imaging (cryo-EM tomography) of samples becomes possible and has been used in conjunction with AFM (see below) to resolve early nonamyloid aggregate structures, e.g., in αSN samples.

In a specialized variant of transmission EM (scanning transmission EM, STEM), the sample is subjected to a subnanometer 100 kV electron beam that scans the specimen in a systematic fashion. This provides scanning EM with analytical capabilities. Thus, the scattered electrons may be recorded, e.g., by energy loss spectrometers to get intensities that are directly proportional to the mass of the irradiated region. In this way protein masses and mass-per-length data may be determined. Also, rather than determining average masses of the combined structural elements in solutions, this allows assignment of masses to specific structural elements in macromolecular assemblies, e.g., Aβ.

Direct visualization of the 3D-structure (topography) of a solid sample in the ambient environment is achieved by atomic force microscopy (AFM). A cantilever with a nanometer radius-of-curvature-sized tip under piezoelectrical control scans the surface of a sample either continuously in contact (static mode) or as an oscillating probe (noncontact or tapping mode) (Figure 6). The tapping mode is well suited for biological samples that may be probed while being in buffer solutions. Cantilever deflections are recorded by optical interferometry, laser reflection, or other means and combined into a 3D-image of the specimen. Especially the height measurements are precise in AFM and thus EM assessment of the width of the aggregates in a sample may be a useful supplement to AFM data. Topographical features are detected with a subnanometer resolution, and by time-lapse studies it is possible to study morphology and growth of aggregates,
nanostructures, protofibrils, and fibrils of, e.g., Aβ.\textsuperscript{103} The main disadvantage of AFM is the limited scan area compared to TEM and SEM (\(\approx10^9\) vs \(10^6-10^7\) \(\mu\text{m}^2\)) and slow scan speed (several minutes vs seconds). Also, as is the case with all imaging techniques there is the possibility of obtaining nonrepresentative ultrastructural images if too few scans are performed.

**NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY**

Nuclear magnetic resonance (NMR) spectroscopy is a versatile and powerful spectroscopic technique for probing structures of isolated homogeneous species in protein aggregation pathways.\textsuperscript{104-108} NMR methods can be divided into solution state NMR and solid state NMR. Both methods may provide structural information about secondary (e.g., identification of β-strand segments and quantification of backbone torsion angles), tertiary (e.g., alignment of β-strands in parallel or antiparallel β-sheets), and quaternary (e.g., relative orientation of β-sheets) structure.

NMR spectroscopy exploits the fact that many atomic nuclei behave like small electromagnets when placed in a strong external magnetic field, i.e., the nuclei have a magnetic dipole moment and the net magnetization vector of individual nuclei may be flipped relative to the external magnetic field by briefly (typically a few microseconds) applying a radiofrequency magnetic field to the sample. By using different and often complex radiofrequency pulse sequences details of the monomeric structure, dynamics, and folding kinetics of purified proteins can be obtained and residue-specific structural changes during self-assembly can be followed. As an example, analysis of the chemical shift and peak intensity of Met35-reduced and oxidized variants of Aβ monomers during oligomerization showed the importance of the oxidative state of Met35 in amyloid formation.\textsuperscript{109}

NMR relaxation is an important solution NMR approach which can provide information on the dynamics and kinetics of the aggregation events. The relaxation times of the net magnetization are monitored when it relaxes back into its equilibrium after having been flipped by different radiofrequency pulses. The relaxation times depend among other factors on the mobility of the protein and the internuclear distances, thus providing details on both dynamics and structure. Detailed information of the exchange kinetics between monomeric Aβ and oligomeric Aβ (protofibrils) was obtained by NMR relaxation\textsuperscript{110} and by a novel NMR spectroscopy variant called dark-state exchange saturation transfer (DEST) NMR.\textsuperscript{111} The latter technique revealed that the exchanging species between monomeric Aβ and protofibrils consist of an ensemble of low populated species with parts that are in either direct contact or tethered to the protofibrils.\textsuperscript{111}

Further, NMR relaxation in combination with stopped-flow fluorescence indicated that transient Aβ dimers were formed prior to oligomerization following binding of Cu\textsuperscript{2+}, and that these metal-induced Aβ dimers may constitute an off-pathway oligomerization step.\textsuperscript{112}

The main disadvantage of solution NMR spectroscopy is the relatively low concentration sensitivity typically requiring protein concentrations of 0.1–1 mM. Thus, it is often necessary to isotope-label the protein. The relatively high concentration requirement is a distinct disadvantage when working with aggregation-prone proteins since higher order oligomers and aggregates cannot be detected by solution NMR. This fact may, however, be exploited in relaxation and DEST NMR studies (cf. above). In addition, data analysis such as peak assignment and relaxation analysis can be very time-consuming for large proteins, and the size limit in solution NMR experiments is typically around 40 kDa.

Solid state NMR (ssNMR) is primarily used for structural determination of insoluble amyloid fibrils, which cannot be analyzed by X-ray crystallography since they do not crystallize. The relatively homogeneous structure of the fibrils provides good quality data, and ssNMR can provide detailed structural information (see above).\textsuperscript{113} In addition, aggregation of the sample is obviously not a problem in ssNMR, and since the fibrillar state is often considered to be the final stage in the aggregation pathway, ssNMR may be less susceptible to dynamic structural changes in the sample. ssNMR has been applied on several proteins associated with neurodegenerative disease, including Aβ,\textsuperscript{114} αSN,\textsuperscript{115} and prion protein.\textsuperscript{116} Interestingly, ssNMR has been used to study the structure of Aβ fibrils induced by isolated amyloid (i.e., seeded growth) from human brain tissue.\textsuperscript{117} It was found that the structure of the seeded fibrils differed from purely synthetic Aβ fibrils. As with solution NMR, peak assignment and data analysis in ssNMR can be very time-consuming, and structural constraints on the molecular model determined by complementary techniques such as X-ray fiber diffraction (e.g., to verify β-sheets), and ultrastructural imaging techniques (fibril dimensions and mass-per-length) can greatly simplify the task of constructing comprehensive models of fibril structures.

**IMMUNOCHEMICAL METHODS**

Antibodies are widely used to demonstrate and quantitate specific proteins in complex biological samples and the tunable properties of antibodies have also been useful for specific detection of protein oligomers and conformers of relevance for neurodegenerative diseases. The fact that a monoclonal antibody usually only has a single binding site (the epitope) on each target antigen may be exploited to construct solid-phase sandwich type immunoassays for oligomers of the target antigen by using the same antibody for capture and detection (cf. Figure 7). In this way only dimeric or higher order oligomers will provide the binding sites necessary for antibody recognition unless the self-assembly leads to occlusion of the antibody epitope. This is the basis for studying Aβ oligomer formation under physiological conditions and in biological fluids using N-terminal or midsequence specific monoclonal antibodies.\textsuperscript{118} A variant scheme is to use two different detection antibodies specific, e.g., for the Aβ N-terminus that are labeled with two different fluorophores. Detection is achieved by fluorescence correlation spectroscopy and a confocal laser scanning microscope in dual color mode. Fluorescence intensity distribution analysis may be achieved by scanning the sample volume precisely by two lasers. Only coincident signals from both labels are counted, and this enables the detection of single aggregates. Other advantages are low sample volume (20 \(\mu\text{L}\) of CSF) and high specificity since three Aβ specific antibodies, one for capture and two (with overlapping epitopes) for detection, are used. In this way it was shown that CSF from AD patients contains significantly higher numbers of Aβ aggregates than CSF from controls without AD.\textsuperscript{118,119} Also, the method has been used in model experiments to visualize αSN aggregates.\textsuperscript{120}

Other immunochemical approaches depend on antibodies developed with and specific for conformational variants,
pathological significance of protein aggregation. In addition, more complete analysis of the role of accessory molecules representing endogenous receptors, inflammatory reactants, chaperones, and proteolytic enzymes will be necessary to enable a better understanding of pathogenesis and thereby to get new angles for combating the chronic neurological diseases associated with protein aggregates.

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- (9) FUTURE DIRECTIONS

Methods for analyzing protein aggregation have developed significantly and cover the whole spectrum from early folding events to deposits of insoluble protein aggregates. While this is applicable for purified species it is not simple to extrapolate from findings in model systems to the events underlying *in vivo* year-long accumulation of protein deposits in neurodegenerative diseases. Hence, in order to understand and thereby combat the chronic neuropathologies, it is paramount to continue developing analytical techniques that are even better at extracting information on protein–ligand and protein–protein interactions and conformational dynamics *in situ* in cell cultures, tissue biopsies, and ultimately in the body. Biological assays (e.g., cell viability) and *in vivo* studies in animal models also play an important role in elucidating the

- **Figure 7.** Quantitative specific immunoassay for a protein oligomer. The assay exploits the fact that only one single antibody binding site exists on the oligomer (the epitope, shown in red) and that the same monoclonal antibody is used both for capture and detection of the protein. Thus, only dimers or higher order oligomers will display monoclonal antibody is used both for capture and detection of the protein. Thus, only dimers or higher order oligomers will display enough binding sites to be captured and detected. Higher order oligomers will yield proportionally stronger signals which also increase with protein oligomer concentration.

- oligomers, or fibrils. In the latter cases, unique linear epitopes only present in oligomerized and fibrillated protein may be envisaged. Conformation-specific antibodies that are specific for polymers of serpins have also been produced, or fibril specific antibodies that will not react with monomers or prefibrillar oligomers or antibodies that react only with oligomers have been developed for Ap. The quantitative capabilities, extreme sensitivity, and the ability to measure specific analytes in complex mixtures are unique for immunoassays but these features completely rely on the availability of antibodies with appropriate characteristics and the analyses are not direct assays. However, the assays may be multiplexed and show great promise in combination with high resolution separation methods, e.g., for the characterization of Ap and αSN isoforms directly from cerebrospinal fluid. Antibodies are also very useful tools for preparatively isolating aggregated protein complexes, e.g., for subsequent characterization of their cargo of aggregated amyloid proteins.
