Microscopy & Microtechniques Focus

ANALYSIS OF AGGREGATED PROTEINS: A CRUCIAL STEP TO UNDERSTAND BSE AND ALZHEIMER'S DISEASE

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Neurodegenerative diseases such as Alzheimer's, Parkinson's and the transmissible spongiform encephalopathies (TSEs) are characterised by abnormal protein deposits, often with large amyloid fibrils. To understand the genesis of these diseases in the case of Alzheimer's or the mechanism of its transmission in the case of TSE a crucial point is to look at the mechanism of the aggregation process that lies at the heart of the damaging effect of these diseases. Classical tools that are well established in protein analysis like HPLC, mass spectrometry, or SEC, are not well suited to study protein aggregation, because of their high molar mass and wide molar mass distribution. Here we present a new method that has been successfully used in research on Alzheimer's or TSE, the combination of asymmetrical Field-Flow Fractionation (AF4) with Multi-Angle Light Scattering (MALS).

> ***THE SIZE OF A MOLECULE IS CALCULATED FROM THE ANGULAR VARIATION OF THE SCATTERED LIGHT INTENSITY***

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THE ANALYTICAL PROBLEM POSED BY PROTEIN AGGREGATION

Protein analysis traditionally has focused on the native, unaggregated protein. Column chromatography is very efficient to isolate the monomer from oligomers and mass spectrometry can be used to identify the material within the peak by establishing the molar mass to a fraction of a Dalton. Aggregates are not considered in this type of analysis, mostly they are removed from the sample prior to injection by filtration or centrifugation; residual aggregates show up as peaks in the exclusion limit and no information can be extracted from them.

In recent years interaction of proteins has become an important topic of research. Interaction of proteins leads to association and aggregation and as a consequence to a mixture of many species with different molar masses. With respect to the monomer molar mass, protein aggregates are huge ranging in the millions of Daltons and often forming protein particles. To characterise these polydisperse mixtures the combination of an efficient separation with detection is needed to generate molar mass distributions capable of resolving species from a couple of thousand Daltons to several million Daltons.

HOW AF4 SEPARATION WORKS

Asymmetrical-flow field-flow-fractionation (AF4) is a non-destructive, one-phase separation mechanism to determine molecular and macromolecular weights and particles in solution. In AF4 components elute at given retention times which are related to the hydrodynamic size of the retained species. This is the case also in SEC, but with column chromatography a calibration with standards is needed to establish the relation between retention time and size. FFF can predict retention for a given component or extract hydrodynamic size straight from a known retention equation and the result of the calculation is the Stokes diameter. The equation only holds in absence of interactions.

Separation is achieved in a channel, consisting of two plates that are separated by a spacer foil; the plates are bolted together *(Figure 1)*. The upper channel plate is impermeable, whilst the bottom plate is permeable and made of a porous frit material. An ultra-filtration membrane with a typical size barrier of 10 kD, covers the bottom plate to prevent the sample from penetrating the channel.

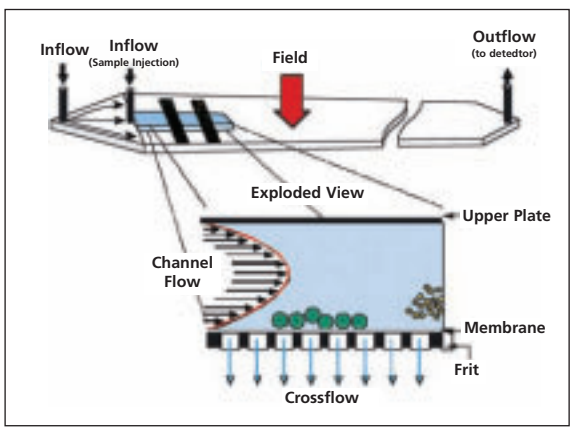

Figure 1. Principle of AF4 separation is shown in the top view of the channel. Below the side view illustrates the separation based on differential placements of sample fractions in the parabolical flow pattern.

Separation needs a sequence of three steps: injection, focusing and elution. During the first two steps the main flow is split, enters the channel from both ends and exits through the bottom porous wall. It is balanced to meet under the injection port. When the sample is injected, it is focused in a thin band and concentrated towards the membrane. The flow permeating through the bottom wall drives components towards the channel boundary ("accumulation wall"). Diffusion associated with Brownian motion creates a counteracting motion, with smaller particles, having higher diffusion rates, reaching an equilibrium position furthest away from the accumulation wall. This process takes typically one minute. For elution, the flow is switched again to enter only at the inlet of the channel and exit at the outlet while the cross flow is maintained at the desired level, that is part of the flow entering the channel continues to exit through the bottom wall.

Because of laminar flow of the liquid in the thin channel, the resulting parabolic velocity profile transports sample components with different speed and therefore separates different sized particles. In the faster flow zones higher up in the channel the smaller particles travel quicker than larger particles which are closer to the accumulation wall. The result is smaller particles eluting before the larger ones, exactly the opposite of a size exclusion or gel permeation (SEC/GPC) separation in which the large molecules elute first.

The process is rapid and non-destructive and the sample remains unaltered by any stationary phase. In comparison to Size-Exclusion Chromatography (SEC), AF4 displays higher selectivity leading to a much broader application range.

MULTI-ANGLE LIGHT SCATTERING (MALS)

MALS detection is crucial to determine molar mass and mass weighted radii at every point of the chromatogram after AF4 separation. The radius calculated is called Root Mean Square Radius (RMS), or rg , which is different from the hydrodynamic radius as it is the average of the mass weighted distance to the center of gravity of every mass point. rg is sensitive to the mass distribution of the molecule or particle. The ratio of rg and hydrodynamic radius rh is characteristic for the conformation of the structure.

To obtain the molar mass the MALS detector needs to be absolute which means calibrated with toluene instead of referencing to a molar mass standard or reliable results cannot be obtained. The size of a molecule is calculated from the angular variation of the scattered light intensity. If a light scattering instrument makes measurements at a single angle only, the angular dependence of scattered light cannot be determined.

Only multi-angle light scattering instruments can be used to determine molecular sizes directly. The MALS instrument needs detectors at a wide range of scattering angles and especially at small angles, because the scattering function can be highly curved; the lowest measurable scattering angle should be at 15°.

Very important is high sensitivity, because extremely low concentrations in the detector cell are typical for this application. Only small samples loads can be injected which are diluted during separation. At the same time the demand is for a high dynamic range of detection. At low angles the scattering intensity can be orders of magnitude higher compared to higher angles and all signals need to be detected with high signal to noise.

ANALYSIS OF PRION PARTICLES

The study on infectivity of prion particles has been published in Nature (Vol. 437, September 8, 2005, doi:10.1038) by Jay Silveira et al at the Rocky Mountain Laboratories, a part of the NIH.

The aim of the work was to evaluate which size fractions are the most infective and if there is a minimum size necessary for infectivity.

To generate a range of aggregate sizes, they used a special method to break down large aggregates of prion particles. The resulting polydisperse mixture was fractionated using the Eclipse AF4 system and sizes determined with a DAWN EOS detector (both from Wyatt Technology Corp., Santa Barbara, USA). A fraction collector was also installed to test for infectivity with a bioassay.

The result was significant: structure below the pentamer do not convert native protein to infective prion aggregates, highest specific infectivity, that is infectivity per unit mass of prion protein, is at a size range of 17-27 nm and molar masses of 300-600 kD.

Large fibrils are not infectious any more. *Figure 2* shows molar mass and size variation across the separated prion particles.

QELS (dynamic light scattering) has been used to calculate the hydrodynamic radius directly. *Figure 3* shows the ration of rg and rh. To higher elution time the ratio increases, which is consistent with the elongated structure of the fibrils formed. Infectivity peaks for particles which are more spherical in shape.

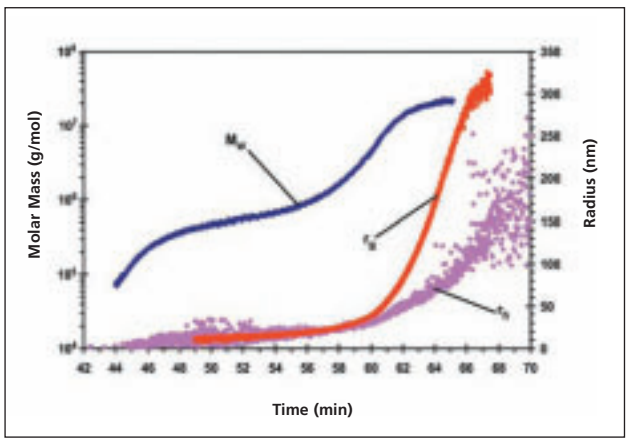

Figure 2. Mass and size analysis of AF4-fractionated prion protein aggregates based on data from 18-angle MALS, DRI and QELS instruments.

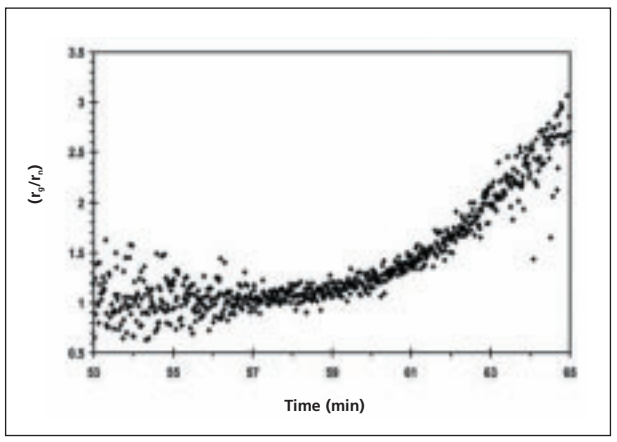

Figure 3. Elution time vs. r value (rg/rh) calculated from the data shown in figure 1, where r values in the range of 0.8 are indicative of compact spherical particles and r values greater 2.0 are typical for extended, rod-shaped particles.