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REPORT: Dynamic Light Scattering Analysis of Sirt3 Protein

Report # PMC122816A prepared by John Philo December 28, 2016, revised December 29, 2016

Purpose

Examine one sample of Sirt3 protein by dynamic light scattering to try to determine its homogeneity and to get a rough estimate for the molar mass of its main component.

Samples

One protein stock described as "Sirt3 protein" at 0.9 mg/mL was provided, along with an aliquot of the corresponding buffer (25 mM Tris + 100 mM sodium chloride + 5 mM dithiothreitol + 10% glycerol, pH 7.5). These materials were shipped frozen on dry ice, received on 12/14/16, and then stored at -80 °C until thawed at room temperature for analysis. These measurements were made on 12/27/16.

Method Background

In dynamic light scattering (also known as quasi-elastic light scattering or photon correlation spectroscopy) the time-dependent fluctuations in scattered light are measured. These fluctuations are related to the Brownian motion of the molecules, and therefore they can be used to determine the diffusion coefficient. This diffusion coefficient is usually converted to the hydrodynamic (Stokes) radius, R_h, through the Stokes-Einstein relation:

$$R_h = \frac{k_B T}{6\pi\eta D}$$

where k_B is the Boltzmann constant, T is absolute temperature, η is the solvent viscosity, and D is the diffusion coefficient.

Typically the light scattering dynamics are analyzed to derive a distribution of hydrodynamic radii. If multiple species are present (*e.g.* aggregates) these may show as a separate peak of larger radius. However, the resolving power is fairly low (it is purely a mathematical resolution, rather than the physical separation present for example in sedimentation velocity). Generally an aggregate must differ in hydrodynamic radius by about a factor of 2 (8-fold in mass) to be resolved as a separate peak. Smaller aggregates such as dimers-tetramers are likely to manifest themselves as a shift of the apparent radius to higher values rather than as a separate peak.

The great sensitivity of light scattering techniques to high molecular weight species is a great strength. However, the consequent high sensitivity to dust or other large particulate contaminants

commonly forces the experimenter to filter the samples, potentially altering the size distribution one is trying to measure (e.g. by breaking up larger particles via shear forces). Our approach is to avoid filtration whenever possible, and when some dust/particulate removal is needed to simply use centrifugation. This approach is greatly aided by the small 1 µL sample volume of our instrument, which greatly reduces the probability of having a dust particle in the sample.

During collection of the dynamic scattering data the average total intensity of the scattered light is also recorded. Separately our instrument also records a batch-mode "static" or "classical" 90º light scattering measurement, using an independent detector. For samples of constant chemical composition this static intensity is directly proportional to the product of the weight-averaged molar mass of that sample times the concentration by weight. For a series of samples at the same concentration their static scattering intensities are therefore a quick and useful measure of the presence of aggregates and the relative weight-average masses of the different samples. (It is possible to calibrate these intensities and get true weight-average masses when that is desired.)

For samples which scatter relatively high amounts of light (for example because the concentration is high) it is important to keep the scattered intensity low enough to avoid saturating the DLS photodetector. For our instrument this is done in two ways: (1) the excitation current in the laser light source can be reduced to reduce its light output; and (2) calibrated attenuators can be inserted between the measurement cuvette and the photodetector. The actual as-measured intensities of the DLS and static detectors are then scaled to account for any attenuation or reduction in incident laser power and reported as "normalized intensities".

Effects of buffer viscosity and refractive index

The measured "raw" hydrodynamic radius values for macromolecules are directly influenced by the fact that buffer components alter the viscosity and refractive index of the solvent. The raw radius values are directly proportional to buffer viscosity (viscous buffers make the macromolecules move more slowly and thus appear larger). The raw radius values are inversely proportional to the buffer refractive index (higher refractive index makes the wavelength of the light smaller, thus making it appear that the molecules move farther per unit time). When the buffer viscosity and refractive index are known the raw radius values can be easily corrected to give the radius that would be measured in water.

Methods

Data were collected at a regulated temperature of 25 ºC using a Wyatt Technology Dynapro Nanostar model WDPN-08 instrument (serial # 325-DPN) using a 1 µL quartz scattering cell.. Typically 25 ten-second data accumulations were recorded and averaged to improve signal/noise. The resulting data were analyzed with the Dynamics version 7.1.8.93 software provided by the manufacturer. Mean (*z*-average) sizes are based on the cumulants method. Size distributions were calculated using the Dynals analysis method, with the resolution set at the default 'optimum' value. Weight fractions were estimated using the Rayleigh spheres model.

The instrument calibration is absolute, based on units of time and distance (with distance measured by the wavelength of the light source). However, that instrument calibration was confirmed on November 18 2016 by the manufacturer during preventative maintenance.

The viscosity and refractive index of the buffer were assumed to be equivalent to those for 10% glycerol within the 1-2% precision of this technique.

Results & Discussion

For any sample the primary result is a graph of the intensity-weighted distribution of hydrodynamic radius. The mean hydrodynamic radius resulting from a simple one-component cumulant analysis will also be reported; this value is essentially a *z*-average radius (weighted to higher mass components), and will only correspond to the true radius for a single-component (monodisperse) sample.[1] The normalized DLS scattering intensity averaged over the data acquisition interval, and the normalized static light scattering intensity (a value proportional to weight-average molar mass) will also be noted. A peak table with the average radius, fraction of total intensity, estimated molar mass (based on globular protein standards), estimated weight fraction, and polydispersity for each peak in the distribution will also be given.

In interpreting the size distribution results it is critical to remember that the scattering intensity of each species is proportional to its molar mass.[2] Thus to translate the intensity distribution (the quantity the instrument actually measures) into a distribution based on weight fractions, one must make an assumption about how molar mass is related to hydrodynamic radius. The assumption always made in DLS is that the molecules are spherical and their molar mass is simply proportional to hydrodynamic radius cubed. With that assumption, the transformation of intensity distributions to weight distributions involves dividing each point in the distribution by the cube of its hydrodynamic radius. Obviously when this is done the results are very sensitive to any error or uncertainty in the R_h of each peak. This makes quantitation of the fraction by weight difficult to reproduce, particularly because some components may contribute significantly to the intensity but only represent parts-per-million by weight. As a general rule of thumb, if the weight fraction of a minor component differs between samples by more than a factor of 2, this might be significant.

An initial attempt to obtain data for the as-supplied sample showed extremely strong scattering (both static and dynamic) that varied substantially in intensity over time scales of seconds to minutes, which indicates the presence of particles with radii larger than ~10 μm. The instrument was also rejecting nearly all the incoming data as unsuitable for analysis. Therefore the stock was centrifuged for 10 min at 10,000 × *g* in a microcentrifuge (Fisher Scientific Marathon 16KM) to remove dust and large particulates prior to loading into the analysis cuvette. This procedure reduced the total scattering intensities about 2-fold.

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[1] For a distribution of molecules, if the *i*-th type of molecule has hydrodynamic radius R_i, weight concentration c_i, and molar mass M_i, then the *z*-average radius is given by $R_z = \frac{\sum_i c_i M_i}{\sum_i \frac{c_i M_i}{R_i}}$

[2] Strictly speaking the linearity with molar mass will only hold true as long as the particle size is quite small compared to the wavelength of light. At sizes of ~100 nm and larger the scattering intensities become strongly dependent on angle (and the detailed particle shape for non-spherical particles).

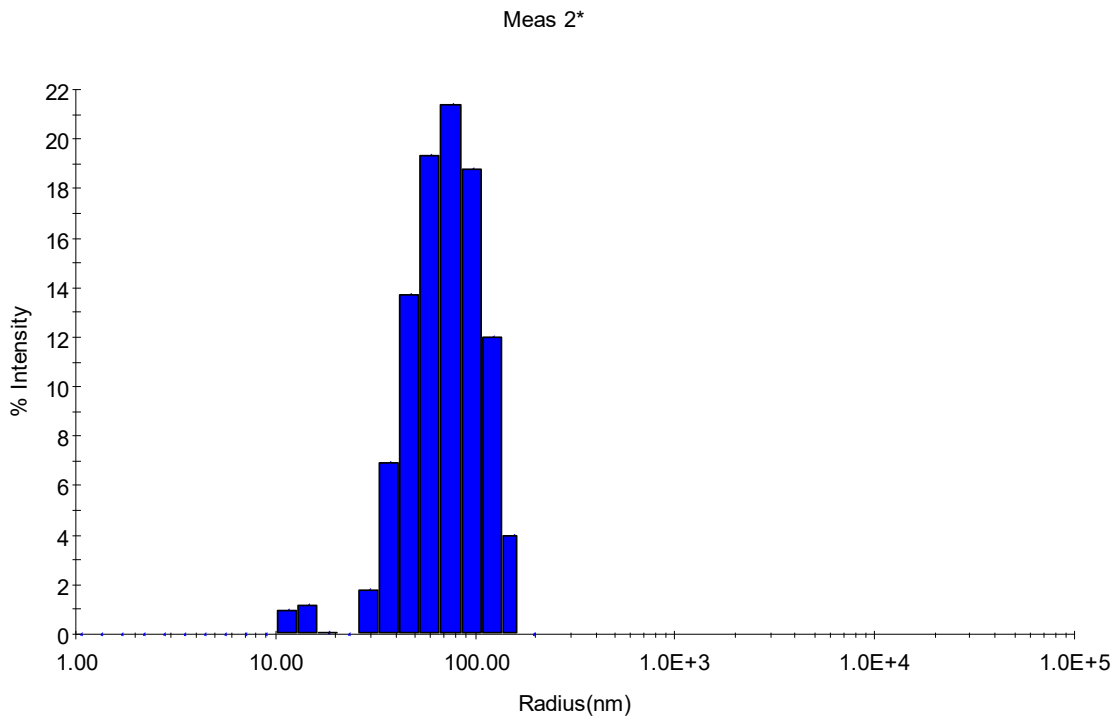

Fig. 1. Hydrodynamic radius distribution for Sirt3 protein. The vertical axis is the fraction of the total light scattering intensity. The horizontal axis is a logarithmically-spaced grid of hydrodynamic radius values (with adjacent points differing by a factor of ~1.3). The analysis covers a range of radii from ~0.01 nm to 100 μm, but any peaks below 1 nm which may be instrument artifacts or due to scattering from salts or other low molar mass excipients have been suppressed.

The size distribution obtained after centrifugation is shown above as Fig. 1. The main peak by intensity, which represents 97.8% of the total scattering intensity, is the second peak, a broad peak which has a mean radius (the intensity-weighted average across the peak) of 78.1 nm. That radius corresponds to a molar mass of 90 MDa, based on globular protein standards. The polydispersity index of this main peak is relatively high at 38.5%, indicating this single peak likely includes contributions from a range of different species.

The first peak has a mean radius of 13.2 nm, which corresponds to an estimated molar mass of 1.4 MDa, and a moderately low polydispersity of 12.7%. Although this peak represents only 2.2% of the dynamic scattering intensity, it is estimated to represent 22.4% of the total on a weight basis. Note that since this peak represents such a small fraction of the scattering its radius is probably not very precisely determined (it may have an uncertainty of 25% or more).

The quantitative results are summarized below, including the *z*-average radius of the entire sample and the normalized scattering intensities, along with a peak table.

Quantitative summary for Sirt3:
z-average radius 63.3 nm; normalized intensities DLS 84.1 Mcnt/s static 11.5 V

Peak #	Mean radius (nm)	Estimated molar mass	Fraction of intensity (%)	Fraction by weight (%)	% Polydispersity
1	13.2	1.4 MDa	2.2	22.4	12.7
2	78.1	90 MDa	97.8	77.6	38.5

Discussion

Is any monomer present?

Our understanding is that the Sirt3 monomer has a molar mass of approximately 30 kDa. No peak with a radius in the range expected for that monomer (about 2.5 nm) was detected, and the lowest radius species detected corresponds to a molar mass of roughly 1.4 MDa. Thus taken at face value the size distribution implies this sample consists entirely of quite large aggregates.

However, because the scattering from the material at ~80 nm is so strong, it is quite possible that smaller species exist, but were not detected because they contribute such a small portion of the total scattering intensity. This is a weakness of DLS that we call "blinded by the light".[3] Generally any peak that represents less than roughly 1% of the total scattering may be suppressed by the regularization algorithms because it is not statistically significant. In extreme cases the low-mass material that cannot be detected may represent 99% or more of the total sample by weight. Thus it is possible that a substantial fraction of this sample is monomer (or at least small oligomers) but was not detected (lost in the glare from the huge peak near 80 nm).

The static light scattering intensity is however not subject to this "blinded by the light" phenomenon, and should always be proportional to the weight-average molar mass of the sample (at least for protein concentrations below ~5 mg/mL). The high static scattering of this sample does indeed imply that the weight-average molar mass (M_w) is fairly large, but may not be consistent with nearly 80% of the total weight fraction being in material in the 100 MDa range. The static intensity of this sample is comparable to that from over 50 mg/mL of an IgG antibody (~150 kDa). Thus given that the protein concentration of this sample is 0.9 mg/mL, its M_w is very roughly $\geq$ 8 MDa. Thus it seems likely that the bulk of this sample on a weight basis cannot be the material with a radius near 80 nm (at masses in the 100 MDa range) as the DLS size distribution implies. Hence it appears likely that a significant amount of lower-mass material is in fact present but could not be detected by DLS. Nonetheless there is no doubt that this sample is quite substantially aggregated.

[3] Philo, J. S. (2009). A critical review of methods for size characterization of non-particulate protein aggregates. *Curr. Pharm. Biotechnol.* 10, 359-372.

Conclusions

1. This Sirt3 sample is highly heterogeneous in size.
2. The dominant peak by both intensity and weight is a broad peak with a mean radius of 78.1 nm, corresponding to very large aggregates in the hundred MDa range.
3. The only other peak resolved, at 13.2 nm, also corresponds to large aggregates of roughly 1.4 MDa.