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Dynamic Light Scattering analysis on RNA associated to proteins.

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Abstract

Dynamic Light Scattering represents an accurate, robust, and reliable technique to analyze molecules size in solution, and monitor their interactions in real time. Here, we describe how to analyze by DLS an RNA-protein interaction. In our frame, we studied complexes formed between RNA fragments derived from the genome of HIV-1 in association with the viral precursor Pr55^{Gag}. These interactions are crucial for the specific selection of the viral genomic RNA (gRNA) from the bulk of the viral spliced and cellular RNAs. This chapter displays how DLS allows to characterize the interactions that regulate the early steps of viral assembly.

1. Introduction

The application of light scattering to analyze biological macromolecules was laid by Rayleigh, Mie and Einstein (*1-3*). Two branches of light scattering were then developed: the Static Light Scattering (SLS) and the Dynamic Light Scattering (DLS). In SLS analysis, the intensity of the scattered light is measured as a function of the scattering angle, which is related to the size and the shape of macromolecules (*4*); whereas in DLS experiments the intensity of the fluctuations of the scattered light is detected as a function of the time, and thus this method provides information about the diffusion, and therefore the hydrodynamic size of the molecules (*5*). Hence, DLS is based on quasi-elastic diffusion of light (QELS) and measures the fluctuation in scattered light intensity over time of molecules diffusing in solution in a random Brownian motion (*6*). The scattered light intensity is then used to generate the autocorrelation function which is related to the diffusion coefficient (D), that provides information on the hydrodynamic radius (R_h) of the molecules via the Stokes-Einstein equation (See the section 3.2) (*7*).

This non-invasive, and reliable method allows typically size measurement of molecules from 1 nm to 10 μm , and whose molecular weight is below 1 KDa. Moreover, DLS is a rapid method providing data in few tens of minutes, and samples do not require labelling or any specific preparation. The analysis is possible at a vast range of solvent conditions, and one only needs knowledge of the viscosity of the solvent, its composition and the related Refractive Index (RI). Compared to other techniques as analytical ultracentrifugation (AUC), the amount of sample required for DLS is quite low (as little as few μl). The measurements can be performed at various temperatures thus providing information about thermal stability of macromolecules. Moreover, DLS is a suitable technique to monitor the complexes formation,

as well as to study the aggregation of macromolecules, which can be useful in preparations of samples for structural studies such as X-ray crystallography (8- 9).

Here we used DLS to analyze the interactions occurring between RNA fragments derived from the viral genome of HIV-1 and the viral precursor Pr55^{Gag}. Indeed, in order to generate an infectious particle, the dimeric unspliced genomic RNA (10) must be selected from more than 100 partially or fully spliced viral RNAs and 10.000 cellular RNAs (11-12). This selection is a highly regulated process relying on specific interactions occurring between the 5' untranslated region (UTR) and the beginning of the Gag coding sequences of the gRNA and the viral precursor Pr55^{Gag}. This last one orchestrates the viral assembly at the plasma membrane, and about 2000 copies of Pr55^{Gag} and the gRNA dimer are encapsidated. The process ends with the budding of the viral particle, which typically have a diameter of about 120 - 150 nm. This study indicates how to compare the interaction of different kinds of viral RNAs (unspliced and spliced) with the Pr55^{Gag} protein by DLS. The characterization of the mean size, the monodispersity and the stability of the different RNA/protein complexes constitutes pieces of information leading to a better understanding of properties required for the proper process of assembly.

2. Materials

1. Prepare all the solutions using ultrapure water (using deionized water attaining a sensitivity of 18M Ω - cm at 25°C).
2. Buffer A: 30 ml of 30 mM Tris-HCl (pH 7.5) with 300 mM NaCl, and 5 mM MgCl₂ (See **Notes 1 and 2**). This buffer is the one used for the DLS analysis of protein-RNA complexes.
3. Buffer B: 30ml of 50 mM Tris-HCl pH 8, supplemented with 1 M NaCl, and 5 mM DTT.

This buffer corresponds to the storage buffer of Pr55^{Gag} recombinant protein (i.e. the absence

of aggregates) (See **Note 3**).

4. 0.22 μm Membrane sterile syringe filter (SARSTED) and 50 ml syringe.
5. Parafilm (Merck Millipore)
6. NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) or equivalent.
7. Single use 50 μl trUView cuvette (BioRad Laboratories) (See **Note 4**).
8. Intensity of the scattered light and correlation times were measured here using a ZetasizerTM Nano S apparatus (4 mW He-Ne laser, $\lambda_0 = 633 \text{ nm}$, scattering angle $\theta = 173^\circ$, (Malvern, UK).

3. Methods

3.1 Samples preparation

1. Buffers need to be filtered as first.
2. Recombinant proteins are stocked at -20°C . Following their thawing, they have to be diluted into a 50 μl solution to a final concentration of 5 - 10 μM in the buffer B, (See **Note 5**), and in the buffer A (See the Material session).
3. Fill the sterile 50 μl trUView cuvettes (BioRad Laboratories) and cover carefully the cuvettes with Parafilm (Merck Millipore) (See **Note 6**). The protein sample is now ready for the analysis (See **Note 7**, and the 3.2.2 Section).
4. Our RNA fragments (600 nts) need to be folded once that the aliquot is thawed. The RNA samples are stored in Milli-Q (Millipore) and then diluted to get the final ratio $\text{Pr55}^{\text{Gag}}/\text{RNA} = 200$. To properly fold the RNA, samples are denatured for 2 min at 90°C and snap-cooled on ice for 2 min. Folding is then achieved by the addition of the binding buffer A and incubation for 15 min at 37°C (See **Note 8**).

3.2 Designing the experiment

3.2.1 Basics

In a typical DLS set-up (**Figure 1**, see **note 9**) the sample is irradiated by a laser and the attenuator is chosen after measuring the scattering intensity on maximum intensity, the typical values should be between 4 and 9. Recommended count rates for DLS measurements are between 100 and 500 kilo counts per second (kpcs). If the sample is too concentrated, and the solution looks white or too hazy, then the measured size of your particles will be inaccurate due to multiple scattering or viscosity effects (See **note 10**). The fluctuations of the of the scattered light are recorded at microseconds time interval and in a typical experiment the software records about 10 to 20 files of about 10 s duration (see **note 11**).

The autocorrelation function is then generated, and its intercept should be about 1, which reflects the quality of the measurements. Besides the decay of the curve is proportional to D and that leads to the determination of R_h which corresponds to the radius of a hard sphere diffusing in solution with the same speed as the particle being measured, or in other words, it corresponds to the size of the solvated particle that is considered as being as spherical. In practice solvated particles can be spherical-like or non-spherical, thus R_h is only an indicator of the apparent size of the molecules or the complexes in solution that are approximated as being spherical. The R_h is related to D by the Stokes-Einstein equation:

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

where T is the sample temperature, η is the solvent viscosity, k_B is Boltzmann's constant ($1.380 \times 10^{-23} \text{ kg} \cdot \text{m}^2 \cdot \text{s}^{-2} \cdot \text{K}^{-1}$).

The data can be represented with intensity, volume, or number-based distributions; however, the intensity distribution is usually chosen since it represents a sensitive detector for the presence of large material in the sample (i.e. aggregates) (See **note 12**). Nevertheless, since DLS is based on Rayleigh theory, the scattered intensity is proportional to the sixth power of the diameter, and thus one has to keep in mind that this analysis results heavily weighted towards larger particle size.

3.2.2 *The experiment*

1. The laser should be switched on 30 minutes before starting the measurement to warm up.
2. The equilibration time prior to the experiment should be set at 2-5 minutes, and in this way the sample should reach the exact temperature suitable for the analysis.
3. Create your own Standard Operating Procedure (SOP). This last one will include the number of the measurements for each sample, the temperature, the kind of the cuvette chosen for the experiment and also the exact composition of the medium (See **note 13**).
4. Fill the cuvette with up to 50 μ l of protein according the kind of cuvettes used for the DLS experiment and check its quality. As explained before 10 to 20 files of about 10 s duration would likely be enough. The distribution should be unimodal (i.e. only one peak) and monodisperse (i.e. very narrow width of distribution, see **notes 14** and **15**). Finally, the autocorrelation functions were analyzed using the DTS software developed by the manufacturer and the estimated molecular weight through the R_h should correspond to the protein molecular weight or proteins multimers as it is in our example. The recombinant protein was tested in the binding buffer (buffer B), and accordingly HIV-1 Pr55^{Gag} precursor in our buffer conditions corresponds to a tetramer (**13**) (**Figure 2**).
5. Then add the previously folded RNA directly in the cuvette to get the final ratio Pr55^{Gag}/RNA = 200 which was chosen to analyze this interaction in conditions approaching the

early steps of the viral assembly. We analyzed the interactions between Pr55^{Gag} and an RNA fragment corresponding to the first 600 nts of the HIV-1 genome, called gRNA. The acquisition led to a corresponding mean R_h of about 170 nm (**Figure 3**). Moreover, the intensity distribution of the complex displayed a unimodal and rather mono-disperse character.

6. We then repeated the same analysis using two others RNA fragments of the same size then gRNA and corresponding to the spliced viral Vpr and Rev RNA respectively (**14**). In this case we observed that the mean R_h was about 380 nm and 425 nm (**Figure 4**). Vpr and Rev RNA interaction with Pr55^{Gag} displayed unimodal intensity distributions but rather poly-disperse. This revealed that most likely several RNA-protein complexes of different sizes were present in solution; therefore this interaction seems to be rather unspecific.

Our DLS test showed this that dimeric gRNA provided more compact complexes compared to the spliced viral RNAs. In addition, DLS analysis over the time (i.e. 2 to 12 h after the formation of the RNA-protein complex) revealed that only the gRNA can form stables complexes in size with the Pr55^{Gag} protein. Even if the size of gRNA-Pr55^{Gag} complexes results to be larger than the typical size of the viral particles, the capacity to stably compact RNA should play a role in efficient Pr55^{Gag} nucleation and in the viral assembly. In the natural context other factors would contribute to stabilize proteins conformation and promote their oligomerization thus leading to proper viral particles formation.

Other techniques as electron microscopy are necessary to provide further information about the shape of those complexes, however in our study DLS offered a reliable characterization of those RNA-protein interactions that regulate the early steps of the HIV-1 assembly.

4. Notes

1. All buffers used here are filtered using a 0.22 μm membrane filter (Millipore) to ensure buffer sterilization, and stored at 4 °C. The buffer solutions are made fresh every week.
2. It is recommended to always rinse the filter prior to use.
3. It is recommended to check the quality of the protein in its storage buffer by a simple DLS test once that it is thawed. In this way one can estimate easily and quickly if the recombinant aliquot of the protein is not aggregated (i.e. the corresponding intensity distribution is not unimodal) or cleaved (i.e. the observed R_h can be related to a sphere whose molecular weight (MW) approaches the expected MW of the protein or the MW of a multimer).
4. It is also possible to use quartz cuvettes instead of single-use ones. In this case to avoid dusts that can become an issue for the analysis, it is important to clean them carefully with the alkaline Hellmanex solution (Hellma Analytics) usually 1-2 volume %. Do not leave the cuvettes in alkaline Hellmanex solution more than one week to avoid quartz deterioration. An increase of temperature speeds up the cleaning process, in this case reduce the application time of the product.
5. To avoid proteins aggregation, it is recommended not to thaw frozen protein aliquots more than once. Also, the DLS analysis in the storage buffer of the protein, which was chosen to preserve the protein, can be considered as a quality control of the samples.
6. Since those cuvettes are devoid of caps, Parafilm avoids dusts to enter in the cuvettes. Dust particles can be indeed a problem, and for this same reason is recommended to filter the samples.
7. The air bubbles in the samples should also be avoided since they can be an issue for the analysis.

8. The RNAs folding is a very important step in our experiences since the secondary structure elements are mandatory for efficient interaction with Pr55^{Gag} protein.
9. In the given example the intensity distribution is represented as a function of the hydrodynamic diameter, d_h , of particles diffusing in solution
10. In our system this value is achieved for micromolar concentrations of protein.
11. It is possible to extend the duration of the records if the molecules analyzed are particularly slow.
12. Since a small amount of larger particle species can dominate the distribution, DLS can place a strong bias on the largest particles in solution,
13. In ZetasizerTM Nano S apparatus the medium composition of the medium can be input in the software, and accordingly the corresponding refractive index (RI) and viscosity values for a given temperature, it will be associated to the experimental data set. The data analysis and the determination of the R_h require indeed the knowledge of those parameters.
14. The Polydispersity Index is dimensionless and is calculated from the cumulant analysis.

In terms of a protein analysis, a % polydispersity less than 20 % indicates that the sample is monodisperse. This % polydispersity can be roughly calculated as the half peak width of the distribution.
15. If the intensity distribution of the protein under observation looks polydisperse (i.e. most likely aggregates are present in solution), it is recommended to ultra-centrifugate the samples for 1h at 45 000 rpm or 100 000× g.

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FIGURES

Figure 1: **A** Schematic of the DLS set-up. The particles in solution are irradiated by a laser beam and the fluctuations of the scattered light intensity are recorded at microseconds time interval. **B** The autocorrelation function is derived, and the decay rate of the curve is proportional to the diffusion coefficient D . **C** This value is then used to determine the hydrodynamic diameter, d_h , which is represented in the intensity distribution.

Figure 2: Pr55^{Gag} samples were characterized by DLS in 50 mM Tris–HCl pH 8, 1 M NaCl. The absorbance of the sample was measured and Pr55^{Gag} concentration was adjusted to 8 μ M. Measurements were performed in a single 50- μ l trUView cuvette (Biorad Laboratories, CA, USA), maintained at 20 °C. Intensity distribution of Pr55^{Gag} samples appeared unimodal and rather monodisperse (polydispersity index=0.22). The mean hydrodynamic radius ($R_h=5.1\pm0.7$ nm) was determined via the Stokes–Einstein equation and this was assigned to Pr55^{Gag} trimers. Our DLS data thus indicated that our Pr55^{Gag} preparations were devoid of microaggregates, which could affect RNA binding studies.

Figure 3: The interaction between gRNA fragment and Pr55^{Gag} was characterized by DLS in 50 mM Tris–HCl pH 8, 1 M NaCl. The ratio Pr55^{Gag}/gRNA= 200 was chosen to mimic the typical conditions of the early events of the HIV-1 viral assembly. RNA-protein complexes were achieved by adding the folded gRNA directly in the cuvette, and the complex was kept at 20 °C. Intensity distribution of Pr55^{Gag} samples appeared unimodal and monodisperse (polydispersity index=0.17). The mean hydrodynamic radius was $R_h=170 \pm 12$ nm.

Figure 4: The interaction between two RNA fragments and corresponding to the VPR (**A**) and REV (**B**) viral spliced RNA and Pr55^{Gag} was characterized by DLS in 50 mM Tris–HCl pH 8, 1 M NaCl. The ratio Pr55^{Gag}/gRNA= 200 was chosen to mimic the typical conditions of the early events of the HIV-1 viral assembly. RNA-protein complexes were achieved by adding the folded gRNA directly in the cuvette, and the complex was kept at 20 °C. The mean hydrodynamic radii were $R_h=320 \pm 11$ nm (**A**) and 425 ± 17 nm (**B**). The corresponding intensity distributions appeared unimodal but rather polydisperse (polydispersity index=0.33 and 0.41).

Figure 1

Figure 2

Figure 3

A

B

Figure 4