2. Tanner, N. K., Schaff, S., Thill, G., Petit-Koskas, E., Crain-Denoyelle, A., and Westhof, E. (1994) A three-dimensional model of hepatitis delta virus ribozyme based on biochemical and mutational analyses. *Curr. Biol.* **4,** 488–498.
3. Sambrook, J., Fitsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Nolan, C., ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
4. Grosshans, C. and Cech, T. R. (1991) A hammerhead ribozyme allows synthesis of a new form of the *Tetrahymena* ribozyme homogeneous in length with a 3' end blocked for transesterification. *Nucleic Acids Res.* **19,** 3875–3880.
5. Price, S. R., Ito, N., Oubridge, C., Avis, J. M., and Nagai, K. (1995) Crystallization of RNA-protein complexes. I. Methods for the large-scale preparation of RNA suitable for crystallographic studies. *J. Mol. Biol.* **249,** 398–408.
6. Been, M. D. (1994) Cis- and trans-acting ribozymes from a human pathogen, hepatitis delta ribozyme. *Trends Biochem. Sci.* **19,** 251–256.

39

Establishing Suitability of RNA Preparations for Crystallization

Determination of Polydispersity

Adrian R. Ferré-d'Amaré and Jennifer A. Doudna

1. Introduction

Successful crystallization of ribozymes and ribozyme domains depends on covalent homogeneity of the sample, conformational homogeneity of the preparation, and an efficient and broad sampling of solution conditions where crystals might nucleate and grow. Chapter 38 presents methods to prepare multimilligram quantities of pure RNA, and Chapter 40 describes strategies for determining crystallization conditions. This chapter presents two methods to determine the conformational homogeneity (polydispersity) of RNA preparations. These methods have been successfully used to determine which of several related constructs of a given target molecule are likely to crystallize, enabling the experimenter to focus on the most promising candidate, and constitute an important step in a successful strategy for crystallization.

The first method is polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions. The equipment needed for its implementation is readily available in laboratories engaged in RNA research, and the technique is familiar to many experimenters. Native PAGE, however, is limited to evaluation of polydispersity under low-ionic-strength conditions and is relatively time-consuming. Its principal drawback is that it involves separation, not examination of the sample directly in solution. Size-exclusion or gel-filtration chromatography, a technique not considered in this chapter, suffers from the same limitation. The second method presented is examination of RNA samples by dynamic light scattering (DLS) *(1)*. This nondestructive technique allows determination of the conformational homogeneity of RNA in solutions containing a wide

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range of electrolytes and additives in a few minutes, but requires access to suitable instrumentation. The commercial availability of compact, microprocessor-controlled DLS instruments and the excellent empirical correlation *(2,3)* between monodispersity as determined by DLS and crystallizability have made the technique very popular in laboratories engaged in macromolecular structure determination *(4,5)*. A detailed example of the interpretation of DLS data collected for evaluation of crystallizability is given in Note 8.

2. Materials

2.1. Native Gel Electrophoresis

1. 40% Acrylamide stock: dissolve 76 g of acrylamide and 4 g of bis-acrylamide in water to give 200 mL of solution.
2. 10X Tris-HEPES-EDTA (THE) buffer: 100 m*M* Tris-HEPES, pH 7.0, 1 m*M* EDTA.
3. 1 *M* $MgCl_2$.
4. Gel electrophoresis apparatus, including gel plates (we use plates 10 × 11.5 in. and 9 × 11.5 in.), 0.5 mm thick spacers and comb, gel box, power leads, and power supply.
5. 10X Native loading buffer: 1X THE, 10% (w/v) sucrose, 0.4% xylene cyanol, and 10 m*M* $MgCl_2$.
6. 37°C Water bath.
7. 65°C Water bath.
8. 90°C Water bath or heat block.
9. UV transilluminator.

2.2. Dynamic Light Scattering

1. DLS instrument (e.g., dp-801, Protein Solutions, Inc., Charlottesville, VA; *see* Note 1).
2. Anotop-10 200 Å pore syringe filters (e.g. Whatman; *see* Note 2) and syringes.
3. 2 mg/mL Solution of bovine serum albumin (BSA) in PBS (*see* Note 3)
4. 0.1% (w/v) SDS in deionized distilled water.
5. Deionized distilled DEPC-treated water.
6. Annealed RNA sample from Section 3.1.
6. 10 m*M* $MgCl_2$, 50 m*M* Mes-KOH, pH 6.0.
7. Centricon microconcentrator (Amicon) or similar device.

3. Methods

3.1. Native Gel Electrophoresis

The procedure described here involves first annealing the RNA sample in buffer and magnesium, and then analyzing the mobility of the sample on a nondenaturing polyacrylamide gel containing magnesium in the matrix and buffer.

1. Prepare four samples for each RNA to be tested as follows. Into each of four tubes, pipet 1–2 µg of the RNA and 1X native loading buffer in a final volume of 10 µL. Tube 1, no incubation; tube 2, incubate at 37°C for 10 min and let cool to room temperature; tube 3, incubate at 65°C for 10 min and let cool to room temperature; tube 4, incubate at 90°C for 5 min and place at room temperature.
2. Prepare a 10% native polyacrylamide gel containing 10% 29:1 acrylamide:bis-acrylamide gel solution, 1X THE buffer, and 10 m*M* $MgCl_2$. The gel should be ~0.5 mm thick, with lanes 0.5–1 cm wide.
3. Set up the gel apparatus with 1X THE buffer containing 10 m*M* $MgCl_2$ as the running buffer. Begin electrophoresis at 10 W, and load the samples as the gel is running. Run until the xylene cyanol dye reaches the middle of the gel (approx 4–5 h).
4. Stop the electrophoresis, open the plates, and cover the exposed gel with plastic wrap. Invert, remove the other gel plate, place gel, still on the plastic wrap, in a glass dish, and cover it with ethidium bromide solution (10 µL of 10 mg/mL ethidium bromide solution in ~200 mL of water). Swirl gently on rotating shaker for 10 min.
5. Visualize the RNA bands by placing the gel on a UV transilluminator (*see* Note 4).

3.2. Dynamic Light Scattering

The procedure described here is suitable for the dp-801 DLS instrument. The RNA sample should first be annealed and brought into the solution conditions under which it is to be examined (*see* Note 5). For this example, the RNA is annealed at pH 6.0 in a moderate concentration of magnesium chloride with no supporting electrolyte or additives, and examined under these conditions. The DLS instrument should be prepared for use by aligning its laser and cleaning the fluidics (or "plumbing") if this has not been done recently. Then the sample examined. The dp-801 will output a table of experimentally determined apparent diffusion coefficients, derived quantities, and residual errors in the analysis of the experimental data. Some example outputs and their interpretation are given in Note 8.

1. Anneal the RNA. At least 150 µL of annealed RNA should be prepared (*see* Note 6). The minimum ribozyme concentration (*see* Note 7) necessary for the experiment depends on the molecule's size: as a rough guide, 3 mg/mL for a relative mol wt (M_r) of 10,000; 2 mg/mL for M_r 20,000; 1 mg/mL for M_r 40,000. If the molecule oligomerizes, its apparent size will be larger, necessitating a lower concentration. Annealing is described in Chapter 40. For this example, the RNA will have been annealed in 10 m*M* $MgCl_2$, 50 m*M* Mes-KOH, pH 6.0 (*see* Note 5).
2. Align laser. Inject 150 µL of a 2 mg/mL solution of BSA in PBS into the dp-801 through an Anotop-10 200 Å filter using a suitable syringe (a 1 mL disposable syringe or a 250 µL Hamilton syringe). Start the "count rate" display, and turn the laser alignment knob slowly in either direction until the number of counts is maximized. Do not move the instrument after this. Flush out the protein with 1 mL of deionized water.
3. Clean the fluidics of the instrument by injecting 1 mL of 0.1% SDS solution (without an Anotop filter) into the instrument and letting it stand inside for 1 h. Thoroughly flush the instrument with deionized water after this.

4. Inject 1 mL of 10 m*M* $MgCl_2$, 50 m*M* Mes-KOH, pH 6.0, through an Anotop filter. Start the "count rate" mode of the instrument, and slowly inject 150 µL of the sample through an Anotop filter. There should be no more resistance to flow than when injecting BSA. The displayed count rate should exceed "20" for accurate measurements. Exit the "count rate" mode, and start "acquire data." The instrument will output analysis results approximately every minute. Collect 5–20 measurements.
5. To recover the sample, flush the fluidics with 1.5 mL of 10 m*M* $MgCl_2$, 50 m*M* Mes-KOH, pH 6.0, and collect the effluent. The ribozyme can now be concentrated in a Centricon concentrator and used for further experiments if desired.
6. If more samples are to be analyzed, prime the fluidics with the appropriate buffer, and repeat steps 4 and 5, above. At the end of the session, flush the instrument with 2–3 mL of deionized water.

4. Notes

1. DLS instrumentation can be constructed from standard electronic and optical components, but operation of such a setup can be technically demanding. The interested reader can consult refs. *(6)* and *(7)*.
2. It is very important to use 200 Å filters to remove dust particles. These filters are also useful to remove excess dust from samples destined for crystallization, if excessive nucleation is a problem. If the ribozyme sample does not traverse the 200 Å filter, it is very heavily aggregated and unsuitable for crystallization.
3. A BSA solution particularly suitable for this purpose because of its reproducibly low monodispersity is the 2 mg/mL solution in glass ampules sealed under argon (oxidation results in polydisperse BSA) distributed by Pierce.
4. RNA samples appropriate for crystallization should typically be at least 95% "pure" by native gel analysis. For samples that have crystallized in our hands, the vast majority of the RNA runs as a single species on a native gel.
5. Typically an RNA construct is first examined after annealing in the presence of 1–15 m*M* $MgCl_2$ and 20–100 m*M* of a buffer of pH 5.0–6.6, in the annealing solution. *See* Chapter 40 for a detailed annealing protocol. If the sample is not monodispersed, DLS can be performed under conditions that are known to be relevant to the activity of the ribozyme. For instance, Group II introns can be examined in the presence of high (~1 *M*) concentrations of, e.g., KCl and 100–200 m*M* $MgCl_2$, and Group I introns in the presence of saturating concentrations of GTP. It is a good idea to examine ribozymes with and without their substrates and inhibitors, if known. In general, "well-behaved" molecules tend to be monodispersed under a variety of conditions.
6. Sample volumes of as little as 50 µL can be used in the dp-801, in principle, since the flow cell has a volume of ~7 µL. However, delivering such a small volume across the syringe filter and through the fluidics into the flow cell is very challenging. Volumes of ~100 µL can be examined by injecting the sample and then "chasing" it with ~100 µL of air, which is itself pushed with some buffer. The air avoids dilution of the sample. If experiments are attempted with such small volumes, it is also worth preceding the sample with a volume of air for the same reason. Note however that any air bubbles left in the flow cell will result in uninterpretable scattering. Having a larger sample volume is also convenient if a bubble does develop, since delivering a few additional tens of microliters of sample will usually dislodge the bubble from the flow cell.
7. The concentrations under which samples are examined by DLS are typically 2- to 10-fold lower than those used for crystallization experiments (*see* Chapter 40). Lack of aggregation under these relatively dilute conditions appears to be an excellent predictor of lack of aggregation at higher concentrations.
8. DLS allows measurement of the translational diffusion coefficient (D_T) of a macromolecule undergoing Brownian motion in solution through analysis of the intensity fluctuations of laser light scattered by the solute. If there is a single macromolecular species, the experiment provides a direct measurement of D_T. Analysis of the autocorrelation function can also provide data regarding sample polydispersity. The technique is exquisitely sensitive to aggregation, since the intensity of scattered light is proportional to the square of the mass of the solute particle. The shape and density of the solute are typically unknown, so the dp-801 calculates an equivalent hydrodynamic radius of gyration (R_H) from D_T using the Stokes-Einstein equation, and a mol-wt estimate using an empirical calibration curve obtained from assorted globular proteins of known mass. The output hydrodynamic radii and molecular weights are approximations only, and can differ substantially from real values for nonglobular, or partially or totally unfolded, or anomalously hydrated macromolecules. For the purpose of assessing crystallizability, however, the values of D_T, R_H, and M_r are of little significance. What matters is that these values are uniform over several measurements, indicative of monodispersity. The illuminated sample volume is on the order of a few microliters, and the presence of even a small number of aggregates will become apparent as large fluctuations in D_T.

Examples of dp-801 outputs obtained from three different related RNA constructs are given in Table 1. The three RNAs share a common structural core, with flanking sequences progressively deleted from "A" through "C." The constructs were first examined for biochemical activity, and then their suitability for crystallization was evaluated by DLS. Construct "A" is highly aggregated, as evidenced by the fluctuation in the apparent values of D_T, R_H, and M_r. The polydispersity values (which represent the standard deviation of the distribution of R_H) are also quite large. Because of this, molecule "A" is unsuitable for crystallization, at least under the solution condition examined here. The apparent M_r is much larger than expected from the covalent molecular weight of the RNA (≈30 kDa). This could be owing either to oligomerization/aggregation or to an increased hydrodynamic drag resulting from an unfolded portion of the molecule. As mentioned above, if the apparent M_r is constant, its disagreement with covalent molecular weight should not be of concern.

Deletion of 16 and 19 nucleotides, respectively, to yield molecules "B" and "C" results in progressively "better behaved" molecules. Molecule "B" still

Table 1
Examples of dp-801 DLS Outputs[a] for Three Related RNA Constructs

Sample	D_T, $10^{-13}m^2/s$	R_H, nm	Polydispersity, nm	M_r, kDa
RNA "A" 89 nt				
	284	5.0	1.954	144
	293	4.9	1.416	136
	291	4.9	1.900	139
	295	4.8	1.714	135
	292	4.9	1.923	138
RNA "B" 73 nt				
	775	2.7	1.049	35
	713	3.0	1.188	43
	741	2.9	1.140	39
	780	2.7	0.969	35
	831	2.6	0.756	30
RNA "C" 70 nt				
	895	2.5	0.710	28
	899	2.5	0.735	28
	889	2.5	0.744	29
	909	2.5	0.704	28
	898	2.5	0.741	28

[a]The results of five successive measurements from each sample are shown for comparison.

shows significant fluctuation of D_T, R_H, and M_r, but displays smaller polydispersities (and smaller R_H/polydispersity ratios). This molecule also displays an apparent M_r, which is in good agreement with the monomeric molecular mass. Molecule "C" is an example of a monodisperse RNA, a promising candidate for crystallization. The values of D_T, R_H, and M_r are stable, the R_H/polydispersity ratio is small, and the apparent M_r is indicative of a monomeric, globular molecule.

After being subjected to a single round of sparse-matrix setups (*see* Chapter 40), molecule "C" yielded crystals, whereas "A" and "B" produced none. It is, of course, possible that an exhaustive screening of crystallization conditions might yield conditions where "B" or even "A" crystallizes. However, given that all three molecules share the same core structure, the path of least resistance to a three-dimensional structure is focusing on the monodispersed construct.

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References

1. Schmitz, K. S. (1990) An *Introduction to Dynamic Light Scattering by Macromolecules*. Academic, San Diego.
2. Zulauf, M. and D'Arcy, A. (1992) Light scattering of proteins as a criterion for crystallization. *J. Crystal Growth* **122,** 102–106.
3. Ferré-D'Amaré, A. R. and Burley, S. K. (1994) Use of dynamic light scattering to assess crystallizability of macromolecules and macromolecular assemblies. *Structure* **2,** 357–359.
4. D'Arcy, A. (1994) Crystallizing proteins—a rational approach? *Acta Crystallographica* **D50,** 469–471.
5. Ferré-D'Amaré, A. R. and Burley, S. K. (1997) Dynamic light scattering as a tool for evaluating crystallizability of macromolecules. *Methods Enzymol.* **276,** 157–166.
6. Wilson, W. W. (1990) Monitoring crystallization experiments using dynamic light scattering: assaying and monitoring protein crystallization in solution. *Methods* **1,** 110–117.
7. Mikol, V. and Giegé, R. (1992) The physical chemistry of protein crystallization, in *Crystallization of Nucleic Acids and Proteins: A Practical Approach* (Ducruix, A. and Giegé, R., eds.), IRL Press, Oxford, pp. 219–239.