Video Article

Monitoring Equilibrium Changes in RNA Structure by 'Peroxidative' and 'Oxidative' Hydroxyl Radical Footprinting

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Abstract

RNA molecules play an essential role in biology. In addition to transmitting genetic information, RNA can fold into unique tertiary structures fulfilling a specific biologic role as regulator, binder or catalyst. Information about tertiary contact formation is essential to understand the function of RNA molecules. Hydroxyl radicals ($\cdot\text{OH}$) are unique probes of the structure of nucleic acids due to their high reactivity and small size.¹ When used as a footprinting probe, hydroxyl radicals map the solvent accessible surface of the phosphodiester backbone of DNA¹ and RNA² with as fine as single nucleotide resolution. Hydroxyl radical footprinting can be used to identify the nucleotides within an intermolecular contact surface, e.g. in DNA-protein¹ and RNA-protein complexes. Equilibrium³ and kinetic⁴ transitions can be determined by conducting hydroxyl radical footprinting as a function of a solution variable or time, respectively. A key feature of footprinting is that limited exposure to the probe (e.g., 'single-hit kinetics') results in the uniform sampling of each nucleotide of the polymer.⁵

In this video article, we use the P4-P6 domain of the *Tetrahymena* ribozyme to illustrate RNA sample preparation and the determination of a Mg(II)-mediated folding isotherms. We describe the use of the well known hydroxyl radical footprinting protocol that requires H_2O_2 (we call this the 'peroxidative' protocol) and a valuable, but not widely known, alternative that uses naturally dissolved O_2 (we call this the 'oxidative' protocol). An overview of the data reduction, transformation and analysis procedures is presented.

Video Link

The video component of this article can be found at <http://www.jove.com/details.php?id=3244>

Protocol

1. Preparation of Footprinting Reagents

1. Prepare a 10x reaction buffer containing 100 mM sodium cacodylate, 1 mM EDTA, and 1 M KCl. Adjust the pH to 7.4. Filter the buffer using a 0.2 μM acetate filter device (Nalgene). Remark: do not pipet RNA directly into 10x buffer.
2. Prepare the titration reaction mix for each reaction as indicated in Table 1. The volume of the titration mix (1x buffer and Mg(II) at the desired concentration) should be 90 μl , before adding 10 μl of RNA in 1x buffer.
3. Prepare a RNase T1 digestion buffer containing 6.63M Urea, 20mM sodium citrate, 1mM EDTA, 0.25 $\mu\text{g}/\mu\text{l}$ tRNA, 0.025% xylene cyanol, and 0.025% bromphenol blue. This buffer can be stored at 4 °C for up to 6 months.
4. The Fenton reaction requires freshly prepared aqueous solutions of 250 mM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, 250 mM EDTA, and 500 mM sodium L-ascorbate. The concentrations noted in chapter 1.5 are optimal for equilibrium experiments in buffer such as sodium cacodylate that does not scavenge radicals and diverse divalent ion concentrations.
5. Reagents for the peroxidative (1.5.1) and oxidative (1.5.2) footprinting reaction.
 1. The peroxidative Fenton reaction requires freshly prepared aqueous solutions of 100 mM Fe-EDTA solution, and 50 mM sodium L-ascorbate and 1.5% H_2O_2 . The Fe-EDTA solution is prepared by mixing $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ with EDTA to have 1.1 times excess of EDTA over iron.
 2. Just before initiating the oxidative cleavage reaction (3.4.2), create the Fenton reaction mix by combining 2 μl of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (250 mM), 2.2 μl of EDTA (250 mM), 62.5 μl sodium L-ascorbate (500 mM), 22.5 μl 10x reaction buffer and 135.5 μl of H_2O to reach the final concentrations in the footprinting reaction mixture (3.4.2) of 0.10 mM, 0.11 mM, and 6.61 mM, respectively.

2. RNA Preparation for Footprinting Experiment

1. RNA can be produced by standard in vitro transcription of DNA templates⁶ or, if shorter than 50 nucleotides, be purchased (e.g. at Integrated DNA Technologies, www.idtdna.com). Follow recommended laboratory cleanliness procedures to maintain sample integrity of RNA during footprinting experiments.⁷

- Dephosphorylation⁸ of in vitro transcribed RNA is necessary prior to ³²P labeling of the 5' end of RNA. 10 pmol of RNA are end-labeled by kinasing.⁹ The solution should be cloudy after successful inactivation of phosphatase.
- Purify radioactively labeled RNA using denaturing gel electrophoresis. The RNA is recovered by two subsequent gel extractions using 0.3 M sodium acetate (pH 5.5). RNA is precipitated by mixing 0.5 ml aqueous solution with 1 ml ethanol and subsequent incubation for 1 minute on dry ice. Spin sample at 4 °C, 12500 rpm for 0.5 hr. Discard the supernatant and wash RNA pellet with 70% ethanol. Remove supernatant after additional centrifugation and dry pellet in vacuum.
- Dissolve the ³²P-labeled RNA samples in 330 µl of 1x Buffer. Pipet 30 µl of the RNA solution in a reaction tube, precipitate with ethanol and dry RNA in vacuum; keep this tube for generating the reference ladder (step 3.5). Denature the buffered RNA solution by heating at 95 °C for 2 min. Cool the samples to room temperature for 15 min and give a quick spin to bring down the condensates on the lids of the tubes back into solution.

3. The Footprinting Experiment

- The electrophoresis comb determines the maximum number of data points per analytical gel. We use a 30 well comb. The reference ladder occupies 2 wells and the uncleaved control one. Prepare 27 reaction tubes. The final Mg(II) concentrations should be evenly spaced on a logarithmic scale spanning orders of magnitude around the titration midpoint.
- Separately, incubate the RNA and the Mg(II) solutions at 50°C for 5 min. Mix 10 µl of RNA solution with 90 µl of the corresponding amount of Mg(II) in 1x buffer to reach a final volume of 100 µl. Incubate the solutions for 30 min at 50°C.
- Let the solutions equilibrate at 25°C for 1 hr while folding occurs.
- Peroxidative(3.4.1) or oxidative (3.4.2) hydroxyl radical footprinting reaction.
 - Start the peroxidative reaction by placing droplets of 2 µl Fe-EDTA (100 mM), 2 µl sodium L-ascorbate (50 mM) and 2 µl H₂O₂ (1.5%) separated from each other at the top inside of the reaction tube containing the RNA solution and start the footprinting reaction by vigorous mixing. Stop the reaction after 15 seconds by adding 300 µl cold absolute ethanol. Turn the tubes 3-5 times. Precipitate, wash and dry the pellet as in step 2.4.
 - Start the oxidative hydroxyl radical footprinting reaction by adding 5 µl of the freshly prepared Fenton reaction mix (1.6). Incubate for 30 min at 25°C. Add 300 µl of cold absolute ethanol to quench the reaction and mix by turning the tubes 3-5 times. Precipitate, wash and dry the pellet as indicated in step 2.4.
- Generate a RNase T1 digest reference sample according to standard procedures¹⁰ using the solution prepared in step 1.3.
- Dissolve RNA pellets in 8 µl gel loading dye II (AMBION) and confirm RNA is resuspended by using the Geiger counter.
- Prepare a denaturing, 8% polyacrylamide sequencing gel according to standard protocols.¹¹ Load samples including two references and a control. Separate RNA fragments at 60 W - 75 W for 2.5 hr. Expose dried gel to a storage phosphor screen overnight. Scan phosphor screen with an imaging system for filmless autoradiography, e.g. Storm 865 (GE Healthcare) or Typhoon (GE Healthcare). Transfer gel image file to your computer.

4. Data Analysis

- The hydroxyl radical reactivity of each nucleotide is determined by quantifying the intensity of each band on the analytical gel. Download, install, and open SAFA, an easy to use open source software for single band fitting and quantification.^{12, 13} Follow the user manual instructions. Briefly, load the RNA sequence as .txt file followed by the gel picture as .gel file. Adjust band intensities, define the lanes, choose an anchor lane, and perform a gel alignment. Assignment of bands to nucleotides occurs in reference to RNase T1 digestion ladder. Quantify the band integrals and use the normalization/colorplot feature to normalize and assign potentially invariant residues. Save the output as a .txt file.
- SAFA outputs a spreadsheet containing columns representing lanes on the gel and rows representing the integrated band density of the individual bands corresponding to the RNA fragment. First, identify the protection sites which display a noticeable change in solvent accessibility by comparing the protection profile derived from the no Mg(II) sample with the profile of the end point Mg(II) concentration (50 mM in our example). The lower the value, the more protected the nucleotide is against the attack from hydroxyl radicals and vice versa.
- Create folding isotherms from the normalized band integrals of individual or groups of nucleotides versus Mg(II) concentration. The isotherms are individually scaled to fractional saturation by $f_i = L + (U - L) \cdot \bar{Y}^i$ where f denotes the integrated density of the band(s) being analyzed, L and U represent the lower and upper limits to the transition and $\bar{Y}$ is fractional saturation.¹⁴ The data are fit using a non-linear least square analysis program (We use either Origin (OriginLab) or GraphPad (GraphPad Software, Inc.)) to the Hill equation (1),

$$\bar{Y} = \frac{K_d^{-n_H} [M]^{n_H}}{1 + K_d^{-n_H} [M]^{n_H}}$$

where K_d is the equilibrium dissociation constant, $[M]$ corresponds to the concentration of the variable that mediates the folding reaction, Mg(II) in this example, and n_H is the Hill coefficient. This procedure scales the transition to fractional saturation, determines its midpoint and provides a phenomenological test of whether a transition is sigmoidal. Isotherms derived from sequencing gels (Figure 3A) that were analyzed by SAFA (Figure 3B) and fit as described above are shown in Figure 3C. The fractional saturation isotherms shown in Figure 3 were generated by scaling in a spreadsheet the values generated by SAFA against the best fit upper and lower limits (U and L) using the equation $\text{fraction} = \text{value} / (U - L) - L / (U - L)$.

5. Representative Results:

Figure 3 shows representative results from P4-P6 RNA •OH footprinting experiments. The gel image (left) indicates that a) background cleavage is minimal (right lane), b) single nucleotide assignment is possible due to well defined bands in the T1 lane (RNase T1 cleaves at each G), and c) the hydroxyl radical induced fragmentation of RNA is well above background. The transition from low to high Mg(II) is affiliated with decreasing integrated band density of individual and groups of bands indicating formation of RNA tertiary structure. Single or groups of contiguous nucleotide whose cleavage changes concomitantly are referred to as a 'protection'. The •OH protections characteristic of the Mg(II) mediated folding of the P4-P6 RNA closely correspond to the solvent inaccessible regions of the molecule observed in its crystal structure.¹⁵

$$\text{A } [\text{Fe(II)-EDTA}]^{2-} + \text{H}_2\text{O}_2 \rightarrow [\text{Fe(III)-EDTA}]^- + \cdot\text{OH} + \text{OH}^-$$

Ascorbate

Figure 1. Hydroxyl radical production and P4-P6 RNA folding. A) Fe(II) catalyzes the generation of hydroxyl radicals from hydrogen peroxide. Ascorbate reduces Fe(III) back to ferrous iron. B) In the absence of Mg(II), no P4-P6 tertiary structure is formed, allowing hydroxyl radicals to penetrate and cleave all accessible backbone positions. Addition of the Mg(II) initiates folding of P4-P6, allowing only the solvent accessible backbone to be cleaved by the hydroxyl radicals.

Figure 2. Outline for a hydroxyl radical footprinting experiment. A) Dephosphorylation and ^{32}P 5'-end labeling of RNA. B) Purification of ^{32}P -labeled RNA on a denaturing polyacrylamide gel. C) Excision of RNA band, subsequent RNA extraction, and ethanol precipitation. D) Prefolding and folding of RNA. E) Addition of freshly-prepared Fenton reaction mixture to generate the hydroxyl radicals. F) RNA fragment separation by denaturing polyacrylamide gel electrophoresis. G) Quantitation of RNA fragments by SAFA software.

Figure 3. Analysis of RNA fragments after peroxidative Fenton footprinting reaction. (A) The RNA was exposed to hydroxyl radicals and the cleavage products were resolved using denaturing polyacrylamide gel electrophoresis (PAGE). The picture shows peroxidative cleavage products with increasing concentration of Mg(II). Reference and control lanes are labeled. This image file is the input for the SAFA program which quantitates the volume of each band. (B) The 'thermal' plot generated by SAFA. (C) A spreadsheet of values relating integrated band density to nucleotide number output from SAFA is analyzed by a binding model such as the Hill equation (1). Regions of protection were chosen according to the increase in band integral density as a function of Mg(II) concentration. The K_d is the Mg(II) concentration at which half of the RNA is folded at the site being monitored. The Hill coefficient, n_H , is a measure of the slope of the curve which gives information on the binding cooperativity and provides a lower estimate for the number of magnesium ions involved in folding of this particular site.

reaction #	final Mg(II) concentration	water	10x buffer	10mM MgCl ₂	100mM MgCl ₂	1M MgCl ₂	RNA in 1x buffer	final volume
2	0	81	9	-	-	-	10	100
4	0.05	80.5	9	0.5	-	-	10	100
13	1.5	79.5	9	-	1.5	-	10	100
22	15	79.5	9	-	-	1.5	10	100

Table 1. Representative volumes for generating RNA samples containing different Mg(II) concentrations.

Discussion

Hydroxyl radical footprinting is a valuable tool to assess the solvent accessible surface area of nucleic acids. Qualitative and quantitative formation of tertiary structure¹⁴ can be followed as a function of parameters such as ion type and concentration, pH, temperature, binding proteins or folding co-factors. The compelling combination of a straight forward and inexpensive protocol and the resulting solvent accessibility and folding information on a single-nucleotide level makes this method very attractive. Traditionally the $\bullet\text{OH}$ footprinting reaction is performed using added H_2O_2 , a protocol we refer to as 'peroxidative' (Figure 1A). The alternative ('oxidative') protocol¹⁷ that we highlight utilizes naturally dissolved O_2 to produce small quantities of hydrogen peroxide via the reactions

Both methods generate $\cdot\text{OH}$ via the oxidation of Fe(II) to Fe(III) by H_2O_2 (Figure 1A). Personal preference seems to determine which protocol is used by members of our laboratory for structural mapping and equilibrium titration experiments. The oxidative and peroxidative footprinting reactions take about 30 and 1 min, respectively. This disadvantage is offset by the oxidative protocol being slightly simpler (one less reagent is added) and the 'crispness' on the oxidative electrophoretic band profiles. While this observation suggests less heterogeneity in the $\cdot\text{OH}$ cleavage products, this hypothesis has not been tested. We recommend using the oxidative protocol to analyze a large number of footprinting samples (> 15) or samples that contain H_2O_2 sensitive components.

The use of the negatively charged EDTA complex of Fe(II) (Figure 1A) prevents association of Fe(II) with the negatively charged phosphate backbone of nucleic acid.¹ Free Fe(II) can bind to DNA and RNA and act as a precipitant and/or can catalyze hydrolysis of the RNA backbone.

The presented concentrations of the Fenton reagents are ideal to cleave P4-P6 RNA under single-hit kinetics conditions necessary for footprinting in the indicated buffer. Single hit kinetics ensure that the RNA molecule is cleaved only once on average.¹⁸ If an excessive amount of Fenton reagent is used, RNA products can arise from more than one cleavage event. This biases the fragment distribution towards shorter cleavage products. The extent of radical cleavage must be adjusted so that ~20% of end-labeled RNA is cleaved and ~80% of end-labeled RNA remains intact. Dose-response experiments must be used to identify the appropriate Fe(II)/ H_2O_2 concentrations for achieving single-hit kinetics under the solution conditions of an investigator's study.¹⁹

The relative solvent accessibility maps obtained from $\cdot\text{OH}$ footprinting analyses can be used alone to characterize the RNA structure, folding and intermediate structures formed during equilibrium folding experiments. These data can be combined with results from techniques addressing changes in global conformation^{7, 20} and/or enzymatic and chemical footprinting studies²¹ which were generated under identical conditions. Coarse-grained models of RNA structures²² can be developed using hydroxyl radical and complementary chemical and enzymatic footprinting profiles as constraints.

Disclosures

No conflicts of interest declared.

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