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Chapter 8

Separating and Analyzing Sulfur-Containing RNAs with Organomercury Gels

Elisa Biondi and Donald H. Burke

Abstract

Polyacrylamide gel electrophoresis is a widely used technique for RNA analysis and purification. The polyacrylamide matrix is highly versatile for chemical derivitization, enabling facile exploitation of thio-mercury chemistry without the need of tedious manipulations and/or expensive coupling reagents, which often give low yields and side products. Here, we describe the use of [(*N*-acryloylamino)phenyl]mercuric chloride in three-layered polyacrylamide gels to detect, separate, quantify, and analyze sulfur-containing RNAs.

Key words: RNA, Thio-phosphate, Sulfur–mercury interaction, APM, Gel electrophoresis

1. Introduction

RNA can acquire sulfur by several means, both within cells and through manipulation in vitro. The modified bases of tRNA constitute the major source of natural sulfur-containing nucleotides in nature, such as thio-substitution of the keto oxygen on positions 2 or 4 of uridine bases and other derivatives (1). 4-thio-U is photoreactive, and is used in RNA–protein cross-linking studies, such as Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) (2). Substituting one of the nonbridging phosphate oxygens with sulfur produces a phosphorothioate. Importantly, for the method presented here, the sulfur atom within phosphorothioates carries a stable negative charge, which greatly increases its chemical reactivity. Phosphorothioate substitutions in catalytic RNAs, such as the hammerhead ribozyme, have been used to analyze the catalytic activity and folding stability of these ribozymes (3, 4), and to determine whether metal ions are coordinating to specific internucleotide linkages (5, 6). Oligonucleotides with

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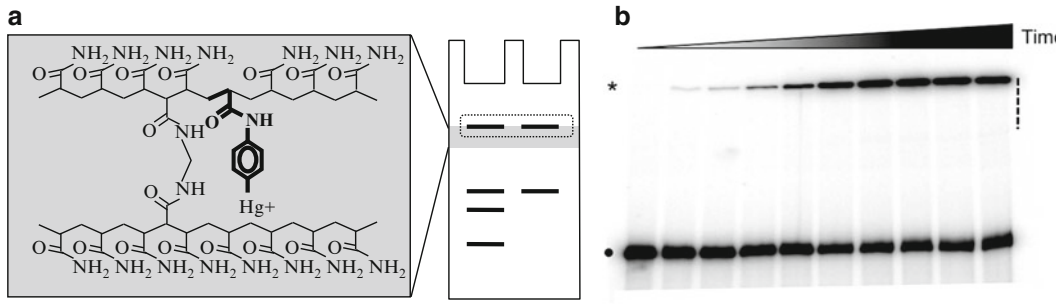

Fig. 1. Three-layered APM gel. (**a**) *Left,* schematic of a polyacrylamide matrix with one APM unit (*in bold*). *Right*, schematic of RNA migration through a three-layer gel. Thio-RNA accumulates at the interface between the top layer and the APM-containing layer (*shaded*), from which it can be excised (*dotted box*) as described below. (**b**) An example of using a three-layer APM gel to analyze accumulation of self-thio-phosphorylated RNA product (*asterisk*) by kinase ribozyme K28(1-98) (23) over time. Non-reacted RNA passes through the APM layer (*dot*). *Dashed vertical line on the right* indicates the height and position of the APM layer (100 μg/mL).

multiple internal phosphorothioate substitutions are under investigation as potential therapeutic agents, in part because of their reduced sensitivity to nucleases (7), and internal phosphorothioate substitutions are critical for nucleotide analog interference mapping (NAIM) to determine the roles of specific chemical moieties in RNA structure and function (8). 5′-thio-phosphorylated RNA is often prepared with the purpose of appending a fluorophore or some other useful compounds at the end of the molecule by virtue of the high reactivity of the sulfur atom, which serves to make maleimide or iodoacetamide conjugates (9). Thiophosphate substitutions have also been widely exploited to reveal RNA–protein contacts, such as the tRNA–protein interactions (10–13). Thus, there are many contexts in which sulfur-containing RNAs can be encountered or generated and for which it can be desirable to have a ready method of analyzing and purifying such RNAs.

Mercury was first used in polyacrylamide gels by Igloi (14) to analyze the content of thio-substituted phosphates in RNAs of different lengths and origins. Mercury is a "soft" metal ion that forms a coordinate covalent bond with the "soft" sulfur ligand, reducing the migration of RNAs that contain sulfur. The mobilities of sulfur-containing RNAs can be highly sensitive to a number of factors. As a result, this method has proven to be very useful for separating RNA molecules on the basis of the numbers of sulfurs they contain or for detecting different positions of the thiols within the RNA molecule (due to position-specific effects in mobility). However, this same sensitivity is problematic in applications, such as purifying all sulfur-containing molecules from non-modified RNAs irrespective of the number and position of the modifications. To this end, we developed methods involving three-layered polyacrylamide gels in which only the middle layer contains a high amount of the organomercurial compound (15) (Fig. 1a). All thio-containing molecules

are retained at the interface between the top layer (normal polyacrylamide) and the middle, organomercurial layer, while RNAs that lack sulfur pass into the bottom layer and are separated on the basis of size. This strategy allows the facile separation of S- and non-S-substituted RNAs. In our lab, we have used this method to separate and quantify the products of in vitro-selected kinase ribozymes' self-thio-phosphorylation reactions (15–24) (Fig. 1b).

2. Materials

Prepare each solution and compound using clean, sterile, RNAse-free labware and Milli-Q water with a resistivity of 18 MΩ. We have not found it necessary to use DEPC-treated water in our RNA manipulations.

2.1. Synthesis of [(N-Acryloylamino) Phenyl]Mercuric Chloride Stock Solution (APM)

1. (*p*-aminophenyl)mercuric acetate.
2. Acetonitrile.
3. 1.2 M $NaHCO_3$.
4. Acryloyl chloride.
5. Dioxane.
6. Formamide.
7. Whatman filter paper.

2.2. Preparation of Three-Layered APM Gels

1. 1–2 mg/mL (0.25–0.50 mM) APM stock solution in formamide.
2. Acrylamide:bisacrylamide (19:1) 40% solution.
3. Urea.
4. 10× TBE (Tris–Borate–EDTA): To a 1-L beaker, add 108 g Tris base, 55 g boric acid, 40 mL 0.5 M EDTA, pH 8.0. Sterilize by autoclaving.
5. 10% Ammonium persulfate (APS) solution: Dissolve 1 g APS in 10 mL Milli-Q water, and sterilize by filtration. Store at 4°C. Do not store for more than 1–2 weeks.
6. *N,N,N',N'*-Tetramethylethylenediamine (TEMED).

2.3. Phosphorimaging and Gel Analysis

No specialized materials required.

2.4. Gel Extraction of Thio-Mercury-Linked RNAs from APM-Polyacrylamide

1. 5 M Ammonium acetate, pH 5.5.
2. 0.5 M EDTA, pH 8.0.
3. 1 M DTT.
4. Pierce Centrifuge Columns, 0.8 mL.

3. Methods

Perform each procedure using clean, sterile, RNAse-free labware and solutions.

3.1. Synthesis of [(N-Acryloylamino) Phenyl]Mercuric Chloride

For the synthesis of the APM powder, we strictly follow the procedure as in ref. 11. In particular:

1. To 0.35 g of (*p*-aminophenyl)mercuric acetate, add 8 mL acetonitrile at 0°C, and stir.
2. Add 2 mL 1.2 M $NaHCO_3$. The solution will start separating into two phases.
3. While vigorously stirring, add 10-µl aliquots of acryloyl chloride up to a total of 0.2 mL added material over a period of 10 min. A white precipitate will form. Let the reaction continue overnight while stirring at 4°C.
4. Remove the white upper phase, which contains the product, and centrifuge.
5. Wash the resulting pellet with water by resuspending the pellet in water and centrifuging again.
6. Dissolve the pellet in 8.5 mL dioxane by warming up to 50°C, and rapidly filter the solution through Whatman filter paper while it is still warm (see Note 1).
7. Leave the solution at room temperature until solid crystals form, then wash the crystals with water, and dry under vacuum.
8. Transfer powder to a pre-weighed empty tube and weigh again to determine yield.
9. Store the APM powder at 4°C.
10. To use in polyacrylamide gels, dissolve in formamide to obtain a stock solution of 1–2 mg/mL. Store this solution at room temperature to avoid slow crystallization of the APM, and use as needed.

3.2. Preparation of Three-Layered APM Gels

This protocol is set for the preparation of medium-sized gels (about 18×25 cm), 0.8-mm thick. For different sizes, just adjust the amounts of solutions and compounds accordingly. Likewise, here, we report the methodology for preparing 10% polyacrylamide and 8 M urea denaturing gel solutions. For different polymer concentrations and/or denaturing strength, adjust accordingly.

1. Prepare 10%, 8 M urea polyacrylamide ready-to-use solution: In a beaker, mix 250 mL 40% acrylamide:bisacrylamide (19:1) solution, 100 mL 10× TBE, and 480 g urea. Stir vigorously, and slowly bring to volume with water while the urea is

dissolving. When the solution reaches a clear color and room temperature, sterilize by filtration. This solution will be stable at room temperature for several months (see Note 2).

2. Assemble the polyacrylamide electrophoresis gel-casting system as for standard gels, making sure to perfectly seal the bottom of the gel cast, which will need to be in an upright position at all times.
3. In a beaker, pour 40 mL of ready-to-use 10% polyacrylamide solution. Add 400 μl 10% APM and 40 μl TEMED, and mix thoroughly (see Note 3).
4. Pour the gel solution into the glass sandwich with the aid of a 50-mL pipettor or straight from the beaker, avoiding trapping of air bubbles in the gel. Fill the gel cast up to about ¾ of the total height of the gel.
5. Let the first layer polymerize in an upright, flat position so as to obtain a flat even layer. Do not allow the interface to dry out (see Note 4).
6. In a small beaker, pour 4 mL of ready-to-use polyacrylamide solution. Add the desired amount of 1–2 mg/mL APM in formamide (for a more precise APM concentration in the layer, make sure to remove from the 4 mL of polyacrylamide solution the same volume of APM you will add) (see Note 5). Mix thoroughly. Add 40 μl 10% APS and 4 μl TEMED. Mix thoroughly. Pour all 4 mL of the second gel layer onto the first layer using a 1-mL pipette, making sure to pour the APM-gel solution from one corner of the gel cast and in an even, smooth, constant flow so as to avoid mercury contamination of other parts of the gel and gel cast.
7. Let the second layer polymerize in an upright, flat position so as to obtain a flat even layer. Do not allow the interface to dry out (see Note 6).
8. Prepare the third and last layer by mixing about 15 mL of ready-to-use polyacrylamide solution with 15 μl TEMED and 150 μl 10% APS. Pour on top of the second layer and add the comb, making sure not to trap bubbles in the wells.
9. Allow the three-layered gel to polymerize for 20–60 min.
10. Pre-run and load the gel as for standard polyacrylamide gel electrophoresis.
11. For good resolution, run the gel at least until any non-thio-RNA present in the samples passes through the middle layer so as to obtain optimal separation.

3.3. Phosphorimaging and Gel Analysis

This protocol refers to ^{32}P-labeled samples, but results can be visualized by several other standard methods for polyacrylamide gel

electrophoresis imaging, such as fluorescence detection and staining with ethidium bromide (see Note 7).

1. Remove the spacers from around the gel and separate the two glass plates by pulling them apart with a spatula.
2. Transfer the gel to plastic wrap (in case the thio-RNA needs to be recovered) or more easily to a used X-ray film (when imaging is all that is needed), paying attention not to rip apart the layers. In case of film, make sure to cover the other side of the gel with plastic wrap.
3. Expose the gel to (unused) film and/or to a phosphorimager screen.
4. The obtained image will look somewhat like the gel in Fig. 1b. The thio-RNAs to be analyzed will appear as bands running exactly on the upper interface of the APM layer, while any non-thiolated RNA present in the sample will run at its expected size in a normal denaturing gel.
5. If desired, ratios of thiolated versus non-thiolated RNAs in the sample can be calculated by quantifying the relative intensities of the bands within a lane, after making sure that the image signal is non-saturated. This can be achieved with any gel-imaging software.
6. If the thio-RNAs need to be recovered from the gel, proceed to Subheading 3.4.

3.4. Gel Extraction of Thio-Mercury-Linked RNAs from APM-Polyacrylamide

1. In a 15-mL conical tube, prepare 10 mL APM-gel extraction buffer: Mix 1 mL 5 M ammonium acetate, pH 5.5 (0.5 M final), 5 mL 1 M DTT (0.5 M final), 0.2 mL 0.5 M EDTA, pH 8.0 (10 mM final), and 3.8 mL water.
2. Cut the band of interest from the gel with a clean blade (see Note 8).
3. Transfer the gel slice to a clean 1.5-mL centrifuge tube, and add 500 μl APM elution buffer. Freeze and thaw the gel slice a couple of times. This partially disrupts the gel matrix and aids elution of the RNA from the gel matrix.
4. Thoroughly crush the gel slice with the silicon plunger's top of a sterile 1-mL syringe, until obtaining a smooth slurry. Note that contrary to normal RNA polyacrylamide gel extractions, in the case of APM-gels, thio-RNA elution cannot be accomplished by overnight incubations at 4°C or other passive diffusion methods due to the strong interaction between sulfur and mercury. Instead, active competition of DTT for the interaction with Hg(II) needs to be achieved by thoroughly crushing the gel slice in the presence of high concentrations of a reductant that contains mercury, such as DTT or β-mercapto-ethanol (see Notes 9–11).

5. Transfer the gel slurry to a filtered column. We use Pierce 0.8-mL centrifuge columns, which carry a polyethylene filter with 30-μm pore size.
6. Place the column in a new 1.5-mL tube, and spin at maximum speed for 2–3 min.
7. Wash the gel slurry left on the column with 200 μl of new APM elution buffer, and then repeat centrifugation.
8. Proceed to ethanol precipitation to concentrate the RNA and eliminate excess salts.

4. Notes

1. In preparing APM, when filtering the pellet dissolved in dioxane, do not use nitrocellulose filters because the organic solvent will dissolve the matrix.
2. Do not store 8 M urea polyacrylamide ready-to-use solutions at 4°C because the low temperature will cause the urea to crystallize in the refrigerator. These solutions are stable for several months at room temperature. To store polyacrylamide denaturing gel solutions at 4°C, limit the concentration of the urea in the solution to 7 M.
3. Although 10% APM solution for polyacrylamide gel polymerization should be prepared fresh as needed, we find that with the ratios we use in the lab (1:1,000) a few 10-mL aliquots can be stored at 4°C for up to a month without compromising good electrophoresis.
4. To avoid letting the gel layer interfaces dry out while casting the gel, it is possible to pour Milli-Q water *very gently* into the gel cast right after pouring the gel layer. Even if it will appear to mix at first, the water phase will soon separate and float on the gel phase, preventing it from drying out and ensuring a flat, even line to form. Once the polyacrylamide layer is polymerized, the water can be easily poured back out of the gel cast.
5. The concentration of APM to be used is different in each experiment. The APM layer can easily be saturated by overloading the wells with thio-RNA or by the presence of other free sulfur-containing molecules (for example, see Fig. 1 of ref. 15). Saturation of the layer will cause your thio-RNAs to fully or partially run through the APM layer instead of being retained at its upper interface. Remember that the mercury–sulfur interaction in a three-layered gel is a 1:1 interaction in a solid phase, confined to a specific space and number of molecules in your layer. The binding capacity is mainly determined by the APM concentration, and by the cross-sectional area of the well

(that is, the thickness of your gel and the width of your wells). In contrast, the height of the middle layer is generally not an issue. To determine which concentration best suits your needs, perform pilot experiments. A good APM concentration range for pilot experiments is 1–100 μg/mL.

6. After pouring the APM-containing layer, droplets of mercury-containing polyacrylamide could get trapped in the still-empty portion of the gel cast, causing a chaotic and spotted retardation of thio-RNAs, and sometimes difficulty in interpreting the results. To avoid this, water can be poured into the gel cast right after the APM-polyacrylamide layer, and then discarded after the layer polymerizes as described above (Note 4). Alternatively, droplets that cling to the glass can be removed through repeated rinses after the APM layer polymerizes. If stubborn droplets of polymerized APM-gel persist, a small piece of used autoradiography film can be used to remove them mechanically.
7. Imaging of three-layered APM gels can be accomplished with virtually any standard method for polyacrylamide gels. Even though radio-imaging ensures higher resolution and sensitivity, the generally low concentration of APM in the gel solution allows for general procedures, such as staining with ethidium bromide or silver.
8. Even though APM gels can be stained or imaged in several different ways, the gel extraction of thio-RNAs retained at the interface of the APM layer can also be performed "blindly." Indeed, in most cases, all the thio-RNAs can be recovered from the gel by cutting a gel slice about 3 mm above and 3 mm below the upper APM interface of the lane in which the sample was run (see dotted box in Fig. 1).
9. Compounds that carry a negatively charged sulfur interact more strongly with mercury than sulfurs that carry partial negative charges or no charge, and the number of sulfurs also influences the strength of the interaction. Thus, an RNA with a thiophosphate (full negative charge) will be bound more tightly and require more aggressive elution than an RNA with a 4-thio-U substitution (no charge on the mercaptoketone sulfur, and only partial sampling of the tautomeric form). Sulfates and disulfides do not interact appreciably with mercury. Similarly, reductants that do not contain sulfur, such as tris-carboxyethyl phosphine (TCEP) (15), are fully compatible with the three-layer APM gel method.
10. While a higher than needed concentration of APM is best to avoid layer saturation when gels are run for the purpose of monitoring and/or quantifying a phenomenon, a lower concentration may be preferable when thio-RNAs need to be

recovered from the APM interface. Indeed, in these cases, it is best to use the minimal concentration of APM that you need to accomplish the separation, as excess mercury rebinds the sulfur atoms in the RNA during the process of DTT-gel extraction and impedes recovery.

11. DTT might cause a problem in subsequent manipulations of APM-gel-extracted RNA. A simple way to get rid of excess DTT in the final sample is to dry the pellet after centrifugation for longer than usual so as to allow for the DTT to completely evaporate. Beta-mercapto-ethanol has a higher vapor pressure than DTT and is removed especially well by this drying method, although it is less effective during APM-gel extraction.

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