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Direct quantitation of Mg²⁺ - RNA interactions by use of a fluorescent dye

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Abstract

The ionic composition of a solution strongly influences the folding of an RNA into its native structure; of particular importance, the stabilities of RNA tertiary structures are sharply dependent on the concentration of Mg²⁺. Most measurements of the extent of Mg²⁺ interaction with an RNA have relied on equilibrium dialysis or indirect measurements. Here we describe an approach, based on titrations in the presence of a fluorescent indicator dye, that accurately measures the excess Mg²⁺ ion neutralizing the charge of an RNA (the interaction or Donnan coefficient, Γ_{2+}) and the total free energy of Mg²⁺ - RNA interactions ($\Delta G_{\text{RNA-}2+}$). Automated data collection with computer-controlled titrators enables the collection of much larger data sets in a short time, compared to equilibrium dialysis. Γ_{2+} and $\Delta G_{\text{RNA-}2+}$ are thermodynamically rigorous quantities that are directly comparable with the results of theoretical calculations and simulations. In the event that RNA folding is coupled to the addition of MgCl₂, the method directly monitors the uptake of Mg²⁺ associated with the folding transition.

I. Introduction

Early RNA folding studies demonstrated the unusual sensitivity of tRNA tertiary structure to Mg²⁺ ions (Stein and Crothers, 1976a); it has subsequently been recognized as a general principle that strong stabilization of an RNA structure by Mg²⁺ is diagnostic of the formation of tertiary contacts (Tinoco and Bustamante, 1999). RNA folding studies have generally measured the net uptake of Mg²⁺ ions upon folding, or used spectroscopic or chemical probes to detect direct ion - RNA contacts (Draper, *et al.*, 2005). To develop a comprehensive framework for understanding the ways in which Mg²⁺ stabilizes tertiary structures, it is necessary to quantitate the ion interactions taking place with both folded and unfolded states of the RNA. The interactions can be described both in terms of the number of excess Mg²⁺ ions neutralizing the RNA charge and the overall free energy of Mg²⁺ interactions. Both experimental parameters are useful benchmarks with which to compare results of theoretical calculations and simulations (Grilley, *et al.*, 2007, Misra and Draper, 2002, Soto, *et al.*, 2007).

Several studies in the 1970s used equilibrium dialysis to follow the excess Mg²⁺ accumulated by transfer RNA (Bina-Stein and Stein, 1976, Stein and Crothers, 1976b). Dialysis is a rigorous way to measure thermodynamic quantities, but its application has been limited by requirements for long-term stability of the RNA solutions and the time needed to

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collect an extensive data set. As an alternative to equilibrium dialysis, chelating dyes have been used to sense the change in Mg^{2+} activity caused by the presence of an RNA. Eriochrome black T, which changes its extinction coefficient upon binding to Mg^{2+} , was used in an set of studies examining Mg^{2+} association with homopolymer RNAs (Krakauer, 1971). The fluorescence of a quinoline derivative has also been used to follow Mg^{2+} interactions with RNA (Römer and Hach, 1975, Serebrov, *et al.*, 2001) and proteins (Pickett, *et al.*, 2003). Here we present methods to measure Mg^{2+} – nucleic acid interactions using a fluorescent dye. The procedure can be largely automated by the use of computer-controlled titrators, enabling the collection of large data sets (>100 points) in less than a few hours. With appropriate controls, the results are as accurate and as thermodynamically rigorous as those from equilibrium dialysis. A single titration data set can be analyzed to find the excess Mg^{2+} associated with an RNA, the free energy of Mg^{2+} interaction with an RNA, and/or the uptake of Mg^{2+} ions accompanying a folding reaction or RNA conformational change.

II. General principles

1. Ion – RNA interactions described by preferential interaction coefficients

The high negative charge of a nucleic acid is neutralized by both an accumulation of mobile cations and an exclusion of mobile anions from the surrounding solution (Record, *et al.*, 1998). The accumulated cations may interact with the nucleic acid in a variety of ways: fully hydrated ions interact strongly with the RNA electrostatic potential at a distance from the RNA surface (the “diffuse ion atmosphere”), cations may be bound to specific sites within the RNA, or cations may contact the RNA surface via an intermediate layer of water (Draper, 2004, Misra, *et al.*, 2003). The depletion of anions near the RNA is caused primarily by repulsive interactions between the ions and RNA negative charges. To describe the accumulation or exclusion of ions in a model-independent way, we use the formalism of interaction coefficients. For Mg^{2+} , the coefficient is defined as

$$\Gamma_{2+} \equiv \left(\frac{\partial C_{2+}}{\partial C_{RNA}} \right)_{\mu_{2+}, C_{\pm}, T, P} \quad (1)$$

where C_{2+} , C_{RNA} , and $C_{\pm}$ are the molar concentrations of Mg^{2+} , RNA, and 1:1 salt, respectively; μ_{2+} is the chemical potential of the Mg^{2+} ion (Grilley, *et al.*, 2006, Record, *et al.*, 1998). Γ_{2+} is the number of Mg^{2+} ions that must accompany every RNA molecule added to a solution, in order to maintain a constant chemical potential of the ion. These Γ_{2+} “excess” ions neutralize $2\Gamma_{2+}$ RNA charges. The remainder of the RNA charge is neutralized by an excess of monovalent ions, Γ_{+} , and a deficiency of anions, Γ_{-} (a negative quantity).

The physical meaning of an interaction coefficient can be illustrated by an equilibrium dialysis experiment, which measures the Donnan coefficient

$$\Gamma_{2+}^D \approx \left(\frac{C_{2+}^{in} - C_{2+}^{out}}{C_{RNA}} \right) \quad (2)$$

C_{2+}^{in} is the molar Mg^{2+} ion concentrations present “inside” the dialysis membrane with RNA (at concentration C_{RNA}). C_{2+}^{out} , also known as the “bulk” concentration of ions, is the molar Mg^{2+} ion concentration “outside” the dialysis membrane. ($C_{2+}^{in} - C_{2+}^{out}$) is the excess number

of Mg^{2+} ions accumulated in response to the presence of the RNA. In a typical set of measurements, a series of RNA solutions would be equilibrated with solutions of increasing Mg^{2+} concentration (and thus increasing μ_{2+}) to obtain Γ_{2+}^D as a function of μ_{2+} . Formally, Γ_{2+}^D differs from Γ_{2+} (equation 1) in that the chemical potential of water is constant across the membrane in a dialysis experiment, while Γ_{2+} is defined for constant pressure. For the concentrations of salt and RNA considered here, the two coefficients are essentially identical and will not be distinguished in the following discussion (Anderson, *et al.*, 2002).

2. Using a Mg^{2+} -binding dye to measure Γ_{2+}

For applications in analytical chemistry, chelators have been synthesized which exhibit large changes in their absorption or fluorescence emission spectra upon binding specific metal ions. In principle, low concentrations of such a Mg^{2+} -binding dye could be used to sense the chemical potential of Mg^{2+} in a solution of RNA, yielding measurements of Γ_{2+} without the lengthy equilibration times needed for dialysis measurements. The experimental approach is sketched in Figure 1A. Sample and reference solutions are prepared with identical concentrations of dye and buffer; the sample solution also contains RNA. The two solutions are titrated with MgCl_2 in parallel, and the fraction of dye molecules complexed with Mg^{2+} , v , is monitored spectroscopically. When v is plotted vs. the total concentration of added MgCl_2 , C_{2+} , a lag is observed in the curve for the sample solution (Figure 1A) because favorable interactions between Mg^{2+} and RNA reduce the effective concentration (or activity) of the Mg^{2+} . The premise of this method, justified below, is that sample and reference solutions with identical values of v also have the same Mg^{2+} ion chemical potential (μ_{2+}) and would therefore be in thermodynamic equilibrium if separated by a dialysis membrane- they would be equivalent to the “inside” and “outside” solutions, respectively. The application of this premise is illustrated by the horizontal arrows in Figure 1A, which compare C_{2+} of the two titration curves at points with the same v value. The Mg^{2+} interaction coefficient is calculated from these concentrations as (cf. equation 2)

$$\Gamma_{2+} = \left(\frac{C_{2+}^{\text{sample}} - C_{2+}^{\text{reference}}}{C_{\text{RNA}}} \right) \quad (3)$$

To justify the assumption made above, that sample and reference solutions with the same value of v would be in thermodynamic equilibrium across a dialysis membrane, imagine a Mg^{2+} - RNA solution that is in dialysis equilibrium with buffer. Samples of the “inside” and “outside” solutions are then spectroscopically assayed with the Mg^{2+} - binding dye and a value for v is obtained after suitable normalization of the absorption or fluorescence signal. If the dye-ion complex has a 1:1 stoichiometry, the fraction of dye molecules complexed with Mg^{2+} is

$$v = \frac{C_c}{C_D + C_c} \quad (4)$$

where C_C and C_D are the molar concentrations of the complex and the unbound dye, respectively. Using the relationships between activity, concentration, and activity coefficient $a_C \equiv \gamma_C C_C$ and $a_D \equiv \gamma_D C_D$, equation (4) becomes

$$\nu = \frac{a_c}{(\gamma_c/\gamma_D)a_D + a_c} \quad (5)$$

The question is whether the same value of ν will be found in both solutions. Because the two solutions are in dialysis equilibrium, a_C and a_D must be identical between them. However, the activity coefficient γ_D or γ_C could differ between the solutions if either the free or complexed dye interacts with the RNA. For the dye used here, the Mg^{2+} -dye complex is electroneutral and thus has no electrostatic interaction with the RNA. As long as there is no direct binding of the complex to the RNA, γ_C is insensitive to the presence or absence of RNA. The dye itself has a negative charge, and thus should be excluded from the RNA-containing solution to approximately the same extent as the chloride ion that is also present. If these experiments are carried out with a large excess of monovalent salt over Mg^{2+} , the overall fraction of negative ions excluded by the RNA is so small as to introduce an error in the measurements of Γ_{2+} that is within the reproducibility of the experiment (see VI below for a calculation of typical error). Thus, under appropriate conditions, $\nu_{\text{in}} \approx \nu_{\text{out}}$ is true to an acceptable level of approximation and the dye can be used to monitor Mg^{2+} activity.

An advantage of using a Mg^{2+} -sensing dye is that one pair of sample and reference titrations yields measurements of Γ_{2+} over a wide range of bulk Mg^{2+} concentrations, as graphed in Figure 1B. This Γ_{2+} curve can then be used to find the Mg^{2+} -RNA interaction free energy by integration,

$$\Delta G_{\text{RNA-2+}} \cong -RT \int_0^{C_{2+}^{\text{bulk}}} \Gamma_{2+} d \ln C_{2+}^{\text{bulk}} \quad (6)$$

$\Delta G_{\text{RNA-2+}}$ is the net free energy change that takes place when MgCl_2 is added to an RNA solution until the bulk Mg^{2+} concentration reaches the upper limit of integration. A detailed derivation of this equation is available elsewhere (Grilley, *et al.*, 2006).

Several approximations have been made in the derivation of equations (3) and (6). The limitations these approximations impose on the measurement of Γ_{2+} and $\Delta G_{\text{RNA-2+}}$, as well as practical considerations in carrying out these measurements, are discussed in section VI below.

2. Interaction coefficients vs. binding densities

Equilibrium dialysis experiments are most often interpreted in terms of a binding density ν , the average number of ligands bound per macromolecule. A plot of ν vs. the “free” or bulk ligand concentration yields a binding curve, which is then fit with parameters describing the stoichiometry and affinities of different classes of binding sites (Wyman and Gill, 1990). Though this approach requires assumptions that are difficult to reconcile with long-range electrostatic interactions of ions, it is nevertheless widely used in the RNA folding literature. Here we contrast the binding density and interaction coefficient approaches, and note why the latter is preferable for the analysis of Mg^{2+} -RNA titrations.

A fundamental conceptual difference between the two approaches is the way in which interaction free energies are described. In a binding formalism, each ligand-bound state of the macromolecule is considered a distinct specie with its own standard chemical potential (μ°). The interaction free energy is then the difference in standard state chemical potentials between the ligand and molecule in free and bound states, e. g.,

$$\Delta G^{\circ} = \mu_{\text{complex}}^0 - (\mu_{\text{macromolecule}}^0 + \mu_{\text{ligand}}^0) = -RT \ln K \quad (7)$$

This approach works well when there are a small number of well-defined binding interactions. It breaks down in several ways when applied to ion - nucleic acid interactions. First, the repulsive interactions between anions and nucleic acids are not readily described in terms of specific “complexes”, and are therefore usually ignored in the binding density formalism. Second, long range electrostatic interactions create concentration gradients of ions near the RNA surface (the so-called “ion atmosphere”); closer cations experience stronger interactions. Because the binding formalism allows any one ion in solution to exist in only one of two states, “bound” in a complex or “free” in solution, these concentration gradients are also ignored. Lastly, the binding formalism relies on formulation of a model that describes all the possible “bound” states of the macromolecule. The usual models for Mg^{2+} - RNA interactions have postulated two or three classes of independent binding sites with “strong” or “weak” affinity (Stein and Crothers, 1976b); some models have included anti-cooperative interactions between ions (Laing, *et al.*, 1994, Leroy and Guéron, 1977). Given enough classes of binding sites, the binding polynomials generated by these models invariably provide a good fit to a data set, but in most cases the equilibrium constants and stoichiometries do not have readily interpretable physical significance, and in any case are not unique descriptions- the data can always be fit by multiple models (Leroy and Guéron, 1977).

When using interaction coefficients, standard state chemical potentials are defined only for each electroneutral component added to the solution. For the cases considered here, there are formally four components: water, an RNA salt, monovalent (1:1) salt, and MgCl_2 . (Single ion chemical potentials may also be defined, subject to the constraint of electroneutrality, e.g. $\mu_{\text{KCl}}^{\circ} = \mu_{\text{K}^{+}}^{\circ} + \mu_{\text{Cl}^{-}}^{\circ}$). Interactions between any two components appear as mutual changes in their thermodynamic activities, which precludes any need to distinguish between bound and free fractions of a component. Thus, in an equilibrium dialysis experiment with Mg^{2+} and RNA,

$$\gamma_{2+}^{\text{in}} C_{2+}^{\text{in}} = \gamma_{2+}^{\text{out}} C_{2+}^{\text{out}} \quad (8)$$

because of the requirement that the Mg^{2+} activity ($a_{2+} \equiv \gamma_{2+} C_{2+}$) be the same on both sides of the membrane. Upon rearrangement, this equation shows how Mg^{2+} - RNA interactions are related to changes in the Mg^{2+} activity coefficient,

$$C_{2+}^{\text{in}} - C_{2+}^{\text{out}} = C_{2+}^{\text{out}} [(\gamma_{2+}^{\text{out}} / \gamma_{2+}^{\text{in}}) - 1] \quad (9)$$

Favorable interactions between Mg^{2+} ions and RNA decrease γ_{2+}^{in} (relative to γ_{2+}^{out}) and thus cause an accumulation of ions by the RNA ($C_{2+}^{\text{in}} > C_{2+}^{\text{out}}$). The left hand side of equation (9) is the numerator of the Donnan coefficient, Γ_{2+}^D (equation 2). Thus, the Mg^{2+} activity coefficient (γ_{2+}) is mathematically related to the interaction coefficient Γ_{2+} , and Γ_{2+} is in turn related to the Mg^{2+} - RNA interaction free energy (equation 6). In contrast to the parameters obtained from a binding density analysis, these three related thermodynamic quantities are model-independent. As such, they may be directly compared with values of

Γ_{2+} and $\Delta G_{\text{RNA-}2+}$ that have been extracted from theoretical calculations and simulations of model systems (Ni, *et al.*, 1999, Soto, *et al.*, 2007).

III. Ion-binding properties of HQS

8-hydroxyquinoline-5-sulfonic acid (HQS) was first described as a soluble quinoline derivative showing substantial changes in its absorption and fluorescence spectra upon chelation of a variety of metals with a valency of +2 or larger (Bishop, 1963a, Bishop, 1963b, Liu and Bailar, 1951). The structure of HQS and its complex with Mg^{2+} are shown in Figure 2A. The sulfonic acid group serves to increase the quinoline solubility in water; with a $\text{pK} \approx 4.0$ (Smith and Martell, 1975), it is fully ionized in the pH range of interest here. The complex with Mg^{2+} has a net neutral charge, because ion chelation is coupled to deprotonation of the quinoline hydroxyl. Free HQS, with a protonated hydroxyl at pH 6–7, has an absorption maximum at 306 nm which is shifted to longer wavelengths (355–357 nm) in the presence of either high pH or saturating concentrations of Mg^{2+} (Tables 1 and 2). Superimposed spectra taken over the pH range 6.0 – 9.5, or with Mg^{2+} concentrations up to 110 mM, show clear isosbestic points (Figure 2B, C). The latter behavior is consistent with the formation of a single, 1:1 Mg^{2+} - HQS chelation complex.

Fluorescence is observed at ~500 nm upon excitation of the 355 nm absorption of the Mg^{2+} - HQS chelate. A ~100 fold increase in fluorescence is obtained upon titration of HQS with MgCl_2 (Figure 3A). Fluorescence intensity data as a function of the total concentration of Mg^{2+} ion, C_{2+} , is fit very well by a single site binding isotherm:

$$I = (I_{\max} - I_{\min}) \left(\frac{K_{\text{HQS}} C_{2+}}{1 + K_{\text{HQS}} C_{2+}} \right) + I_{\min} \quad (10)$$

where $I_{\max}$ is the intensity of the Mg^{2+} -HQS complex, $I_{\min}$ is the intensity of the free HQS, and K_{HQS} is the apparent equilibrium constant for formation of Mg^{2+} -HQS. At the concentration of HQS typically used in these titrations, the fraction of added ions that are bound to HQS is very small and accuracy is not compromised if the total ion concentration (C_{2+}) is used in place of the free (unbound) ion concentration.

K_{HQS} is pH dependent, because chelation of Mg^{2+} by HQS promotes deprotonation of the quinoline hydroxyl group. $\log(K_{\text{HQS}})$ increases linearly with pH over the range 6 – 8 (Figure 3B); the calculated pK_a is 8.43 and the intrinsic K°_{HQS} is $11.3 \times 10^3 \text{ M}^{-1}$, in agreement with literature values (Smith and Martell, 1975). The large fluorescence enhancement and range of Mg^{2+} binding affinities around neutral pH make HQS a suitable indicator for experiments measuring Mg^{2+} -RNA interactions.

Starting from Mg^{2+} and going down the periodic table column of group II ions, HQS-metal ion binding constants become weaker and the fluorescence enhancements become smaller. At pH 8.0 the apparent equilibrium constant for Ca^{2+} -HQS formation is 182 M^{-1} and the fluorescence enhancement is ~75 fold (Figure 3B). The Ba^{2+} complex is even weaker, with an apparent binding constant of 36 M^{-1} and a fluorescence enhancement of ~10 fold. HQS may be a good sensor for Ca^{2+} - as well as Mg^{2+} - RNA interactions, but its affinity for Sr^{2+} or Ba^{2+} is probably too weak for this purpose.

IV. Preparation of solutions and reagents

1. Reagents and stock solutions

Measurement of metal ion-RNA association is critically dependent on the purity of the components that are used. All solutions should be made from pure water with at least 18 M Ω resistivity. Purchased buffers and salts should be at least 99.5% pure, but because EDTA is included in all the buffers to scavenge transition metals, salts of higher purity (and expense) are not necessary.

HQS can be purchased from Sigma Chemicals in a relatively pure acid form. The small amount of metal contaminating the HQS can be removed by recrystallization at acid pH. First saturate ~50 mL of pure water with the dye (~5 g). To this solution add concentrated (12 N) HCl until no further color change or precipitation is perceptible. Heat the solution to boiling while stirring to fully re-dissolve the HQS. Cool the solution on ice and drain the excess water leaving the crystalline HQS as very fine needles. Add fresh pure water and HCl to the HQS crystals, then heat with stirring until the HQS is all dissolved. Cool, drain and repeat the process seven times, adding less water and HCl at each step to account for the slight loss of HQS that remains dissolved at the end of the cooling step. After the final step, add 50 mL water and raise the pH to approximately 7 with KOH. Continue to add water and maintain pH 7 until all crystals are dissolved. When HQS treated this way is diluted into freshly made buffered solution, no change in its absorbance spectra is seen upon addition of 100 μ M EDTA. The concentration of the stock solution can be determined using an extinction coefficient at the 326 nm isosbestic point (Table I) of 2600 M⁻¹ cm⁻¹. The purified HQS can be divided into aliquots (~100 mM stock is typical) and stored in acid rinsed glassware for years.

The 99.99% purity MgCl₂•hexahydrate available from Aldrich can be used as supplied to make ~2 M MgCl₂ stock solutions. MgCl₂ solutions for titrations are prepared by diluting the stock 2 M MgCl₂ solution into the appropriate titration buffer (see IV.1 below).

Titration buffers should be made from weighed salts and the acid form of the buffer; titrate with the appropriate 99.5% pure hydroxide salt to adjust the pH. Some convenient buffer concentrations and pHs for different monovalent cation concentrations are as follows:

- A. 20 mM MOPS pH 7.2, 20 μ M EDTA, 20.0 mM K⁺, 10.0 mM Cl⁻
- B. 20 mM MOPS pH 7.0, 20 μ M EDTA, 40.0 mM K⁺, 32.3 mM Cl⁻
- C. 20 mM MOPS pH 6.8, 20 μ M EDTA, 60.0 mM K⁺, 54.3 mM Cl⁻
- D. 20 mM MES pH 6.15, 20 μ M EDTA, 150 mM K⁺, 140 mM Cl⁻

(Both buffer and EDTA stock solutions were prepared from the respective free acids and adjusted to the desired pH with KOH. The K⁺ concentration is the sum of the weighed KCl and the KOH added to adjust the pH.) Lower pH, which weakens the affinity of HQS for Mg²⁺, is paired with higher salt concentration to match the weakened Mg²⁺-RNA interactions. These pairings have given reproducible measurements of Γ_{2+} with duplex DNA restriction fragments, RNAs that have Mg²⁺-dependent or Mg²⁺-independent tertiary structures, and RNAs with only secondary structure. Titrations at pH 8.0 (and thus larger K_{HQS}) extend the range of useful data to C_{2+}^{bulk} concentrations as low as ~1 μ M (D. Leipply, personal communication).

Glassware, plastic Eppendorf-style micro-centrifuge tubes and other preparative labware leach metals into solution. For that reason, 20 μ M EDTA was included in all titration buffers. It is important to note that this level of EDTA serves only to scavenge transition

metals, which bind EDTA much more tightly than does Mg^{2+} ; an order of magnitude higher EDTA concentration is needed before a significant amount of Mg^{2+} is bound.

The hygroscopic properties of MgCl_2 affect the accuracy with which the salt can be weighed. Therefore either of the following two protocols is used to standardize MgCl_2 solutions. Both are based on EDTA- Mg^{2+} complex formation but use different methods to detect the stoichiometric break point.

The first protocol uses HQS as a reporter of free Mg^{2+} . An accurately-known concentration of EDTA (1 – 5 mM) in 100 mM EPPS (pH 8.0) and 100 μM HQS is titrated with MgCl_2 solution. Either fluorescence or absorbance of the HQS is plotted vs. added MgCl_2 to find the amount of Mg^{2+} needed to titrate the EDTA to a stoichiometric endpoint. pH 8.0 provides an appropriate ratio of the EDTA and HQS binding affinities for accurate determination of the stoichiometry. A high buffer concentration is necessary because of the pH dependence of EDTA- Mg^{2+} binding (Martell and Smith, 1974).

The second protocol depends on the hypochromicity of EDTA in the low UV region (200–250 nm) upon metal ion binding. Titrations are conducted in 10 mM MOPS (pH 7) using semimicro quartz cuvettes and recording an absorbance spectrum between 200 and 350 nm. In a typical experiment, 800 μL of a ~1 mM solution of the metal ion of interest is titrated with 1 to 3 μL aliquots of a 40 mM EDTA solution. Plots of the absorbance at 230 nm versus the concentration of EDTA yield stoichiometric titrations with sharp break points that are used to calculate the concentration of the metal ion.

2. Sample Preparation

Metal-free DNA or RNA fragments can be prepared using a DEAE matrix made by Qiagen, which comes pre-packaged in disposable columns appropriate for small step gradients. A Qiatip-2500 column holds 2.5 mg of double stranded nucleic acid (5 mg single strand), and has a column volume of approximately 30 mLs. The resin can be removed and repacked into a Biorad Econo-column for use with pumps or gradient makers. To use the column first make the following two buffers:

Buffer A: 67mM MOPS, pH 6.8; 533 mM NaCl; 20% EtOH

Buffer B: 67mM MOPS, pH 6.8; 3.0 M NaCl; 20% EtOH

All the solutions that follow are adjusted to a final pH of 7.0 with NaOH after mixing buffer A or B with the other components. The resin is first washed with 0.15% Triton X-100, 25% water, and 75% buffer A, and then equilibrated with 25% water, 75% buffer A prior to loading the sample. Samples are diluted with 3 volumes Buffer A and loaded onto the column. If an RNA transcription mixture is being loaded, add sufficient EDTA to dissolve any magnesium pyrophosphate precipitate. After loading, rinse with 25% water, 75% buffer A until a stable baseline (monitored at ~260 nm) is achieved. The column is then eluted in steps consisting of mixtures of buffers A and B in different ratios and 25% (v/v) urea (e.g., 100 mL of a 600 mM NaCl solution is made by mixing 67 mL buffer A, 8 mL buffer B and 36.2 g urea). Since addition of urea slightly shifts the pH, mix buffers A and B and dissolve urea before bringing the solution to pH 7.0 with NaOH. The Qiagen columns are particularly sensitive to pH. Double-stranded DNA tends to elute between 0.9 and 2 M NaCl, RNA between 0.4 and 1 M NaCl; larger nucleic acids elute at higher salt concentrations. Very large (greater than approximately 400 basepairs) nucleic acids may require higher pH for elution (e.g., 2 M salt and pH 8.0). Nucleic acids should be precipitated out of column fractions by addition of 50% isopropanol, which minimizes precipitation of the salt and urea.

RNA transcripts for titrations can also be purified by denaturing gel electrophoresis followed by electroelution from an Elutrap (Schleicher & Schuell). The high concentration of EDTA and urea used in standard electrophoresis buffers are as efficient in removing Mg^{2+} and transition metal contamination as the above column protocol.

Precipitated, purified nucleic acids should be resuspended in 50 mM EDTA, pH 7.0, 1 M ammonium acetate to help remove any remaining metal contaminants. The nucleic acid can then be exchanged into titration buffer. Amicon centrifugal filtration devices (Millipore) are large enough (4 or 15 mL) to provide a reasonable dilution factor without concentrating the sample too much. Extremely high concentrations of nucleic acid (greater than 40 mM nucleotide) equilibrate slowly and should be avoided. A good final stock concentration is between 10 and 30 mM in nucleotide. Typical equilibrations involve at least eight fivefold dilutions. Nucleic acids approach equilibrium with low salt buffers very slowly by either dialysis or repeated rounds of concentration and dilution. We therefore first attempt to equilibrate samples with buffers containing half of the desired final salt concentration, and then bring the salt concentration up to the final desired concentration in the final dilution/concentration cycles.

The approach of the sample to the final salt concentration can be tracked by UV-monitored melting curves. Sample RNA concentrations are adjusted with equilibration buffer to an RNA appropriate for a titration (1 – 4 mM nucleotide), and melting is monitored by absorbance at an appropriate wavelength in a 1 mm path length cuvette. The same experiment repeated on a sample diluted 10 fold (0.1 mM nucleotide) in 1 cm cuvette should have the same melting profile if equilibration has been achieved. Because of the high concentration of the sample, formation of RNA dimers is a potential artifact that could also cause differences between the melting profiles at two different RNA concentrations.

V. Instrumentation and data collection protocols

1. Automated titrations

All HQS titrations were performed on an Aviv ATF-105 differential/ratio spectrofluorometer designed for computer-controlled titrations into reference and sample cells. The instrument includes a pair of automatic dispensers (Hamilton) and “J”-shaped tubes for titrant delivery into standard 1 cm² cuvettes. This setup works well for a high density solution (such as concentrated urea), but the buffered, 12 mM MgCl_2 titrant solutions used here easily mix with the cuvette solution during set-up. The problem can be overcome by using a glass capillary tube that has been pulled into a very fine tip. The glass tips can be held in fittings with M6 threads by wrapping the tube with Teflon tape (Figure 4). The assembly is held by a teflon cap machined for the purpose (E in Figure 4). This arrangement enhances “wicking” of sample up the cuvette walls and increases evaporation, but these problems are minimized by applying a hydrophobic coating to the walls. (Soak inside of cuvettes with 15 M nitric acid for at least one hour. Rinse well with pure water and dry. Add Sigmacote (Sigma-Aldrich) to the cuvette and let sit for at least an hour. Rinse well with pure water.)

The Hamilton dispensers can be used with syringe sizes from 25 μL to 25 mL. With 1000 steps per stroke, the 25 μL syringe could have a resolution in volume delivery of as little as 0.025 μL . However, the 25 μL syringes tend to wear out more quickly than the larger volume syringes. Thus we use 50 μL syringes as a good compromise between resolution and long-term stability. Regardless, the valves and syringe plungers wear down over time, leading to inconsistent results. With daily use, syringe plungers should be replaced about once a month, the entire syringe about every six months, and the valves every three to four months.

The syringes and valves on the dispensers also leach metals and need to be rinsed with 5 mM EDTA solution prior to each day of use.

In designing an HQS titration, the total time of the experiment and the number and volume of injections are important and related considerations. In general, we have found that the shorter the experiment, the more reproducible the data. For nucleic acid systems in which Mg^{2+} does not induce any folding reaction, the only limitation on the interval between additions is the time taken for mixing, about 15 seconds at moderate stirring speeds. The folding kinetics of some RNAs can require equilibration times of five or more minutes for each Mg^{2+} addition. (A necessary control is to obtain time courses of RNA folding after Mg^{2+} addition in the UV spectrophotometer under similar solution conditions as desired for the HQS-monitored titration; based on such kinetic data, an appropriate interval can be designed for the HQS-monitored titration. Apparent folding rates are usually not uniform as one titrates across a folding transition.) The longest a titration should be is about five hours; longer titrations require corrections for drift in instrument signal (which is exacerbated by fluctuations in room temperature) and titrant and sample evaporation (to allow titrant addition, the cuvettes cannot be completely sealed). It is easier to repeat a titration several times with different nucleic acid concentrations and schedules of Mg^{2+} additions than to try to obtain a complete range of data in a single long titration.

A single titration experiment requires data from two cuvettes run in parallel: a reference cuvette with HQS and titration buffer and a sample cuvette with RNA. The same batch of titration buffer with which the RNA has been equilibrated is used in both cuvettes. When preparing the sample and reference cuvettes, absorption spectra should be taken to check the RNA concentration and test for metal ion contamination. First, scan buffer alone in both cuvettes (550 – 200 nm). Add the nucleic acid to the sample cuvette (typically a five fold dilution) and perform a second scan. The absorbance at 260 will be far beyond the linear range of any spectrophotometer; use the absorbance values between 0.2 and 1.2 OD in the range 295 – 310 nm to check the RNA concentration. Add HQS, typically 1–2 μL of stock to a final concentration of 20 – 50 μM , and perform a final scan. (HQS concentrations as low as 10 μM may be used, if accurate data at bulk Mg^{2+} concentrations in the μM range are to be collected.) Metal ion contamination of the RNA stock solution is evident in the shape of the HQS absorbance curve between 350 and 400 nm (Figure 2). A final diagnosis of contamination may be made by adding EDTA to 100 μM and looking for changes in the HQS spectrum; however, the sample cannot then be used without re-purification.

A typical titration protocol with the automatic titrators uses starting volumes of 2.00 mL in sample and reference cuvettes. The two titrant solutions are:

Sample: Titration Buffer + 12 mM MgCl_2 (60 mM MgCl_2 for pH 6.15 buffers)

Reference: Titration Buffer + 120 mM MgCl_2

The same buffer used to equilibrate samples (see IV.2 above) is also used for the titration buffer in each solution; unbuffered stock MgCl_2 solutions are as described in IV.1. Because titration buffer is slightly diluted when making the sample and reference titrants, there will be a ~0.2% change in salt and buffer concentration over the course of the entire titration; the magnitude is not large enough to be of concern.

A typical titration schedule is as follows (concentrations listed are for the reference cell, but the same volume additions are used for sample cell):

Section:	1	2	3
Final $[\text{MgCl}_2]$	12 mM	20 mM	40 mM

Step size	200 μ M	400 μ M	1 mM
Number of Points	60	20	20

The Aviv fluorometer software calculates the injection volumes needed to achieve constant increments in MgCl_2 concentration; constant volume additions could be used as well. The standard stirring time between additions is 30 sec; nucleic acid samples that fold may require adjustment in the stirring times programmed for each section.

To reduce problems with self-quenching, data are collected on the edge of the HQS absorption maximum by exciting at 405 nm with a 2 nm bandwidth; emission is monitored at 500 nm with an 8 nm bandwidth.

After the automatic titration is finished, manual additions are made to help determine the fluorescence intensity at saturating Mg^{2+} (I_{max} ; see section VI. below). Unbuffered 2 M MgCl_2 is added in the following volumes: 5, 5, 10, 20, 20, 20, and 20 μ L.

2. Manual Titrations

HQS titrations can also be performed by manual additions of Mg^{2+} with standard pipetters. Manual titrations can be carried out in small volume cuvettes, which is useful when the RNA of interest cannot be obtained in large amounts. In a typical titration, a stoppered microcuvette containing 400 μ L of an HQS solution is titrated in parallel to a cuvette containing 400 μ L of HQS and the RNA of interest. We recommend using a 10 μ L pipetter during the entire experiment for consistency. Use three MgCl_2 stock solutions (made in titration buffer): 7, 35 and 1000 mM. Titrate the RNA containing cuvette initially with 7 mM MgCl_2 (45 additions of 1–10 μ L), then with 35 mM MgCl_2 (three 10 μ L additions) and finally with 1000 mM MgCl_2 (five 10 μ L additions). Titrate the cuvette containing only HQS with 35 mM MgCl_2 (48 additions of 1–10 μ L) and 1000 mM MgCl_2 (five 10 μ L additions). Add the same volume of titrant to both the RNA and HQS cuvettes. Monitor the fluorescence intensity until the HQS signal stabilizes, and take six additional measurements of the intensity to average for a single data point.

VI. Data analysis

An example titration data set is shown in Figure 5. The most reliable and informative data are obtained near the beginning of the titration, where the difference between the sample and reference curves is most pronounced. In the example shown, this region corresponds to HQS normalized fluorescence (saturation) values between 0 and 0.15 (cf. the inset in Figure 5).

To begin data analysis, measured intensities for the sample cuvette are first normalized:

$$I_{\text{norm}} = \frac{I - I_{\text{min}}}{I_{\text{max}} - I_{\text{min}}} \quad (11)$$

I_{norm} is the fraction of Mg^{2+} -bound HQS, which will be compared with the reference titration (Figure 1A). I_{min} is taken as the initial data point (before MgCl_2 addition) of the sample cuvette. I_{max} is obtained from the fit of a single site binding isotherm (equation 10) to only the manual titration points of the sample cuvette (Figure 5). More complicated schemes for obtaining I_{max} were tried; all gave identical results within error.

The data from the reference titration are fit to a single site binding isotherm (equation 10), allowing K_{HQS} , I_{max} , and I_{min} values to float. K_{HQS} is then used to calculate the “bulk” Mg^{2+} concentration, C_{2+}^{bulk} , for each data point in the sample curve:

$$C_{2+}^{bulk} = \frac{I_{norm}}{K_{HQS}(1 - I_{norm})} \quad (12)$$

Finally, the preferential interaction coefficient is computed by calculating the excess Mg^{2+} present in the sample cuvette over the calculated bulk Mg^{2+} concentration:

$$\Gamma_{2+} = \frac{C_{2+}^{sample} - C_{2+}^{bulk}}{C_{RNA}} \quad (13)$$

C_{2+}^{bulk} is identical to $C_{2+}^{reference}$ in equation (3); equation (12) simply calculates this value from the fitted reference HQS - Mg^{2+} binding curve.

Errors in four experimental measurements can affect the accuracy of the calculated Γ_{2+} values. These quantities and their estimated typical uncertainties are I_{max} (<1%), K_{HQS} (<1%), nucleic acid concentration (3%), and stock $MgCl_2$ concentration (1%). Figure 6 shows the effect on the calculated value of Γ_{2+} when each of these quantities is perturbed by 3 – 10%. Errors in I_{max} result in small changes in Γ_{2+} for the initial titration points, and much larger errors for the final titration points. Any error in determining the stock $MgCl_2$ concentration will be partly compensated in the calculation of C_{2+}^{bulk} by the fact that an overestimation of the total added $MgCl_2$ decreases the calculated K_{HQS} (and *vice versa*).

Errors in the RNA concentration result in the same percentage change in Γ_{2+} at all C_{2+}^{bulk} . Combining all of the typical estimated magnitudes of errors, their net effect on Γ_{2+} in the Figure 6 data set is similar to the variation observed between titrations repeated under identical conditions in the range from $\sim 3 \times 10^{-6}$ to nearly 10^{-3} M bulk Mg^{2+} .

To average data from separate titrations, we choose a set of $\ln(C_{2+}^{bulk})$ values, calculate the corresponding Γ_{2+} for each data set by linear interpolation between neighboring data points, and average the Γ_{2+} values so obtained. Titrations carried out at different pH values can be averaged to extend the range of C_{2+}^{bulk} values for which errors in Γ_{2+} are acceptable. Three to five independent titrations are typically averaged to obtain a reliable data set; the standard deviations of the averaged values give an estimate of the associated error. Integration of the averaged Γ_{2+} curve to obtain interaction free energies (Figure 1B) is done either by using the trapezoidal rule of numerical integration or by fitting a polynomial that asymptotically approaches the x-axis, $y = b(x-a)^2 + c(x-a)^3 + d(x-a)^4$, and integrating the polynomial. The two methods generally agree within error. In either case, free energies calculated at low $\ln(C_{2+}^{bulk})$ are potentially subject to systematic error from the way the curve is extrapolated to the x-axis.

V. Controls and further considerations

Here we discuss a number of factors that should be taken into account in designing a titration experiment and analyzing the results, either because of practical limitations in the experimental set-up or assumptions that are inherent in equations (6) and (12).

- (i) The possibility that RNA-RNA interactions bias the measurement of Γ_{2+} should be checked by making measurements over a range of RNA concentrations and, if necessary, extrapolating to infinite dilution (Strauss, *et al.*, 1967). We have made measurements in the range of 0.25 to 6 mM nucleotide (~ 2 mg/ml, in 40 mM

monovalent cation) for an RNA without detecting a significant concentration dependence in Γ_{2+} . The potential for RNA aggregation at high concentrations can be minimized by careful design of the sequence (Szewczak, *et al.*, 1990).

- (ii) The dye or Mg^{2+} -dye complex should not bind directly to the RNA. We check to make sure the dye absorbance and fluorescence spectra are not perturbed by RNA, and also compare RNA melting profiles carried out in the presence and absence of dye. We have no evidence for HQS or $\text{HQS}\cdot\text{Mg}^{2+}$ binding to any RNA.
- (iv) Equation (13) is valid only when there is a large molar excess of monovalent salt (e.g., KCl or NaCl) over added MgCl_2 . As discussed above (II.1), HQS can be used as a sensor of the Mg^{2+} activity only if repulsive electrostatic interactions between the RNA and the negatively charged dye negligibly perturb v , the fraction of dye molecules that are in complex with Mg^{2+} (equation 4). The effect of RNA on the measurement of v is estimated as follows. Suppose that an equilibrium dialysis experiment is carried out with an RNA concentration of 5 mM nucleotides. For most RNAs, anion exclusion is on the order of $\Gamma_- \approx -0.1$; thus the difference in anion concentrations between the two sides of the membrane is $C_-^{\text{in}} - C_-^{\text{out}} \approx -0.5 \text{ mM}$. If 50 mM KCl is present, then the ratio of anion activity coefficients on the two sides of the membrane is about $\gamma_-^{\text{in}}/\gamma_-^{\text{out}} \approx 1.01$ (cf. the analogous equation 9 for Mg^{2+}). Because anion exclusion is entirely a result of unfavorable coulombic forces, the identity of the anion is unimportant and HQS^- and Cl^- should be affected similarly. Thus γ_D (equation 5) is $\sim 1\%$ different between the two sides of the membrane, and the corresponding error in the measurement of v is $<1\%$, which is comparable to the experimental error (see section VI above).
- (v) In the derivations of equations (6) and (10), Mg^{2+} ion concentration (C_{2+}) has been substituted for activity (a_{2+}). This substitution is justified only if the Mg^{2+} activity coefficient, γ_{2+} , is a constant over the course of the titration. In a solution of strong electrolytes, γ_{2+} is primarily affected by favorable electrostatic interactions with anions. If a large enough concentration of NaCl or KCl is already present in solution, the Cl^- concentration does not significantly change as MgCl_2 is added, and γ_{2+} is approximately constant. Whether the Cl^- concentration is in large enough excess over Mg^{2+} is checked when a single site binding isotherm (equation 10) is fit to the reference titration data. A non-random distribution of residuals is observed if γ_{2+} is not constant over the range of MgCl_2 concentrations being used (D. Leipply, personal communication). A 10 – 30 fold excess of Cl^- over the Mg^{2+} concentration at the midpoint of the titration generally reduces the error to levels comparable with experimental variation. Note that an excess of monovalent ions is also required in equilibrium dialysis or any other experiment that attempts to extract thermodynamic information from a titration of RNA with MgCl_2 .
- (vi) The derivation of equation (6) assumes that RNA does not undergo any conformational changes as the Mg^{2+} concentration increases. If an RNA adopts additional structure in response to the presence of Mg^{2+} , an inflection may be observed in the plot of Γ_{2+} vs. $\ln(C_{2+}^{\text{bulk}})$; the measured Γ_{2+} is then a weighted average over the RNA species present in solution. If the conformational change is two-state, it may be possible to find the number of Mg^{2+} ions taken up or released in the transition (Grilley, *et al.*, 2006). An RNA may also change conformation during the titration without any inflection appearing in the plot of Γ_{2+} , for

instance if there is gradual compaction of an ensemble of extended RNA structures as Mg^{2+} is added (Grilley, *et al.*, 2007).

- (vii) The HQS titration method can be applied, in principle, over a wide range of monovalent salt concentrations. However, there are some practical considerations. At low salt concentrations, it becomes difficult to assure that equilibrium with the nucleic acid has been achieved in preparation of the sample, and it also becomes difficult to reduce adventitious metal ion contaminants to a sufficiently low level. These limitations set a lower limit of ~20 mM monovalent ions. At high salt concentrations, the weakness of the Mg^{2+} -nucleic acid interactions and the correspondingly high concentrations of nucleic acid that must be used in the experiments become limiting. Measurement of I_{max} and K_{HQS} become difficult because of the high Mg^{2+} concentrations required to saturate HQS. The solution viscosity and concentration-dependent behavior of the nucleic acid may also cause problems. We have measured Γ_{2+} curves to $>1 \text{ mM } C_{2+}^{\text{bulk}}$ for a 58mer RNA with 150 mM K^+ present, which is probably close to the practical limit (Grilley, *et al.*, 2007).

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Figure 1.
Experimental approach for using an indicator dye to measure (A), Γ_{2+} and (B), ΔG_{RNA-2+} for an RNA. See text for a description.

Figure 2.

Properties of HQS. A) HQS reaction with Mg^{2+} ion. B) HQS extinction coefficient as a function of pH (20 mM buffer: MES, MOPS, EPPS or CHES; 40 mM K^+ ; 25° C). The pH values are 6.0 (gray), 6.4, 6.8, 7.0, 7.2, 7.4, 7.6, 8.0, 8.5, 9.0 and 9.5 (black). The absorbance does not change much for pH values less than 7.6. Extinction coefficients at high and low pH are compiled in Table I. C) HQS extinction coefficient as a function of Mg^{2+} concentration. The titration was performed in 20 mM MOPS pH 7.0, 10.0 mM K^+ , 6.43 mM Cl^- . The gray line represents 0 mM $MgCl_2$, and the black line 110 mM $MgCl_2$. Extinction coefficients at saturating Mg^{2+} are compiled in Table II.

Figure 3.

A) Titration of HQS with MgCl_2 , pH 6.8 with a total of 60 mM K^+ . The curve is the least squares best fit to equation (9), with $K_{\text{HQS}} = 372 \text{ M}^{-1}$. B) Effective HQS binding constants as a function of pH: closed circles, Mg^{2+} ; open circle, Ca^{2+} ; open triangle, Ba^{2+} . Error bars represent one standard deviation calculated from at least three independent measurements. The dark line is a fit to the equation shown and gives the proton dissociation constant (K_a , $\text{pK}_a = 8.43$) and intrinsic HQS- Mg^{2+} binding constant ($K_{\text{HQS}}^0 = 11.3 \text{ mM}^{-1}$). The K^+ concentration is 40 mM.

Figure 4.

Computer controlled titrator setup. A) Hamilton Microlab 500 Dispenser, with 50 μL gastight syringe; B) FEP (Hamilton Company) tubing with M6 threaded fittings, one milliliter capacity; C) Union, M6 threads; D) Fittings, M6 thread; E) Polymer cap, machined from teflon with a cavity made to hold "D" and a hole made to accommodate the capillary tube; F) glass capillary tube, held in place with teflon tape, tip tapered and bent to fit into corner of cuvette and just long enough to reach solution, but not interfere with light path; G) cuvette and teflon stir bar.

Figure 5.

An example titration showing reference HQS titration (filled circles) and sample RNA titration (open squares). The fluorescence data have been normalized (equation 10). The data from both the sample and reference cuvette have been fit to Eq. 9 (black and gray curves respectively). The fit to the sample cuvette data uses just the manual titration points, only one of which is visible on this scale (solid square). The inset shows the values of Γ_{2+} calculated from equation 5. Vertical lines (both main graph and inset) show the end of each section of the titration schedule in the sample cuvette. The most informative part of the titration curve for calculating Γ_{2+} is the initial, sharply curved “lag”.

Figure 6. Effect of ± 3 (solid dark gray), ± 5 (dashed medium gray) and $\pm 10\%$ (dotted light gray) perturbations of either I_{max} (A), K_{HQS} (B), RNA concentration (C), or stock $MgCl_2$ concentration (D) on Γ_{2+} and C_{2+}^{bulk} . The arrows indicate the effect of an increase in each value.

Table IHQS absorption properties^a

Maxima at high and low pH			pH-independent isosbestic points	
Wavelength (nm)	ϵ (cm ⁻¹ M ⁻¹)	pH	Wavelength (nm)	ϵ (cm ⁻¹ M ⁻¹)
240	26300	6.0	244	19000
253	24000	9.5	270	3250
270	3250	6.0	326	2600
306	3600	6.0	418	135
357	3700	9.5		

^aSpectra were taken at 25° C in either 20 mM MES pH 6.0, 40.0 mM K⁺, 31.7 mM Cl⁻, or 20 mM CHES pH 9.5, 40 mM K⁺, 30 mM Cl⁻. All extinction coefficients are based on an extinction of 2600 cm⁻¹M⁻¹ at 326 nm determined from 3 separate preparations of HQS in 10.0 mM EPPS, pH 8.0, 5.0 mM K⁺, 0.1 mM EDTA.

Table II

Mg²⁺-HQS absorption maxima and Mg²⁺-independent isosbestic points at pH 7.0^a

Wavelength (nm)	ϵ (cm ⁻¹ M ⁻¹)	type
244	17700	isosbestic
255	24200	maximum
267	3400	isosbestic
329	2250	isosbestic
355	3400	maximum
415	110	isosbestic

^aHQS extinction coefficients were measured in the presence of saturating Mg²⁺ (170 mM MgCl₂, 20 mM MOPS pH 7.0, 10.0 mM K⁺, 6.43 mM Cl⁻). Wavelengths, but not extinction coefficients, of Mg²⁺-HQS absorbance maxima and Mg²⁺-independent isosbestic points are constant with pH.