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Rapid agarose gel electrophoretic mobility shift assay for quantitating protein: RNA interactions

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ABSTRACT

Interactions between proteins and nucleic acids are frequently analyzed using electrophoretic mobility shift assays (EMSAs). This technique separates bound protein:nucleic acid complexes from free nucleic acids by electrophoresis, most commonly using polyacrylamide gels. The current study utilizes recent advances in agarose gel electrophoresis technology to develop a new EMSA protocol that is simpler and faster than traditional polyacrylamide methods. Agarose gels are normally run at low voltages (~10 V/cm) to minimize heating and gel artifacts. In this study we demonstrate that EMSAs performed using agarose gels can be run at high voltages (≥20 V/cm) with 0.5 × TB (Tris-borate) buffer, allowing for short run times while simultaneously yielding high band resolution. Several parameters affecting band and image quality were optimized for the procedure, including gel thickness, agarose percentage, and applied voltage. Association of the siRNA-binding protein p19 with its target RNA was investigated using the new system. The agarose gel and conventional polyacrylamide gel methods generated similar apparent binding constants in side-by-side experiments. A particular advantage of the new approach described here is that the short run times (5–10 min) reduce opportunities for dissociation of bound complexes, an important concern in non-equilibrium nucleic acid binding experiments.

1. Introduction

The efficient separation of bound and unbound molecules is critical for accurate measurement of the binding affinity between a protein and its cognate ligand. Historically, dissociation constants (K_D) for protein:nucleic acid interactions have been measured using both equilibrium-based and non-equilibrium-based methods. Examples of equilibrium-based approaches to measure dissociation constants include techniques such as isothermal titration calorimetry and sedimentation equilibrium [1–4]. Non-equilibrium methods include techniques such as native gel electrophoretic mobility shift assays (EMSAs) and filter-binding assays, which are affordable, fast, and measure *apparent* dissociation constants [5–7]. To most accurately capture the amounts of free and bound ligand at equilibrium, these latter methods require rapid separation of the free and bound species and quantitation of their relative amounts.

Because of their simplicity, low cost, and speed, EMSAs are among the most commonly used approaches for studying interactions between proteins and DNA or RNA. EMSAs also afford the ability to monitor the formation of multiple bound species, including supershifts and cooperative complexes [8]. Typical EMSA experiments involve separation of free nucleic acids from bound complexes using native polyacrylamide gel electrophoresis (PAGE). Polyacrylamide gels have historically been preferred over agarose gels because they exhibit greater resolution of the relatively small nucleic acids frequently used in the assays [9,10]. However, the use of polyacrylamide gels for conventional EMSAs involves several drawbacks. For example, the polyacrylamide gels used to separate free and bound ligands require the handling of unpolymerized acrylamide, a potent neurotoxin. Additionally, nondenaturing polyacrylamide gels must be prepared by performing free radical-catalyzed polymerization reactions that are more tedious than the simpler methods required to prepare agarose gels. Methods that avoid these approaches increase safety and reduce cost to the laboratory. Because of their ease of preparation and use, agarose gels have been employed in a number of past studies involving EMSAs. The first agarose-based EMSAs were generally restricted to larger nucleic acids, ranging in length from hundreds of nucleotides

Abbreviations: EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; $K_{D,app}$, apparent dissociation constant; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; nt, nucleotide.

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[11–17] to thousands of nucleotides [18–21] because of the poor resolution of small oligonucleotides in conventional agarose gels. To improve oligonucleotide resolution, researchers have successfully employed a variety of approaches, including higher percentage agarose gels, the incorporation of a commercial additive, a proprietary agarose matrix, and by altering the running buffer to contain lithium borate in place of conventional Tris or MOPS buffers [22–27]. These studies spanned a variety of protein:nucleic acid systems, including transcription factors, recombinases, transposases, internal ribosomal entry sites, and ribosomal proteins bound to pre-rRNAs. While nearly all of these studies observed distinct shifted bands, they all describe EMSAs that were run for over 30 min, and many experiments required up to 4 h of electrophoresis.

Recently, improvements in agarose gel electrophoresis conditions have been described that allow for rapid (<15 min) separation of small DNA and RNA species with high resolution using high percentage agarose (>2.5%) and 0.5 × Tris-borate (TB) buffer without EDTA [28]. By reducing the buffer concentration and eliminating EDTA, gels could be electrophoresed at higher voltages with less heating and with shorter run times. It is likely that faster runs in EMSA experiments, reducing opportunities for irreversible dissociation while in the presence of an electric field, will permit more accurate measurements of the initial equilibrium binding conditions. Faster run times also reduce diffusion of nucleic acids in gels, resulting in sharper band resolution [28]. Therefore, this method should be especially beneficial for separation of the small, highly-diffusible nucleic acids (<50 nt) that are often used in traditional polyacrylamide EMSAs. The new approach has recently been used to measure the adsorption of 25 nt and 54 nt RNA molecules to clay nanoparticles [29].

We show here that these rapid agarose gel methods can also be adapted for quantitatively measuring binding affinities between proteins and nucleic acids. More specifically, we have optimized the previously described agarose gel electrophoresis approach for use with EMSAs involving binding of proteins to small RNA oligonucleotides. As a model RNA:protein binding system, we employed the siRNA-binding protein p19 and its cognate dsRNA ligand [30]. This stable intermolecular interaction has a reported $K_{D,app}$ of 170 ± 20 pM, determined using conventional polyacrylamide gel EMSAs in conjunction with radiolabeled RNA ligands [31]. The binding affinity of a commercial form of p19 for its cognate RNA was measured using the new high voltage agarose method and the results were compared to those obtained using conventional polyacrylamide gels. The aggregate data demonstrate that EMSAs performed using agarose gels can produce results that are qualitatively and quantitatively comparable to those obtained with PAGE gels.

2. Materials & methods

2.1. Binding reactions

RNA oligonucleotides (p19RNA-1, 5′-AUCUCAACCAGCCACUGCUAA, and p19RNA-2, 5′-AGCAGUGGCUGGUUGAGAUUU) were obtained from Integrated DNA Technologies. The 21-nt complementary strands were annealed by mixing equivalent molar amounts of each oligonucleotide, heating to 90 °C for 4 min, then allowing the solution to slow cool to room temperature over 30 min. Successful annealing was confirmed by agarose gel electrophoresis (Supp. Fig. 1A). The p19 siRNA binding protein used here was a commercially produced double-fusion protein, with a maltose binding protein fused to the N-terminus and a chitin-binding domain fused to the C-terminus (New England BioLabs). The binding properties of this double-fusion version of p19 have been previously described [32]. Twelve-microliter binding reactions were prepared with 20 ng of double-stranded RNA in 1 × Binding Buffer (NEB) with varying protein concentrations. The reactions were briefly vortexed and then incubated at 25 °C for 1 h. After incubation, 2 μL of 30% glycerol was added to each sample and, after brief vortexing, a total of 11 μL was loaded into each well of either an agarose or a polyacrylamide gel. Gels were prepared during incubation so that electrophoresis could immediately follow the binding reaction.

2.2. Agarose gel electrophoresis

Various percentages of agarose gel solutions were prepared with agarose LE powder (Gold Biotechnology) using 0.5 × TB buffer (45 mM Tris, 45 mM boric acid) [28]. To ensure uniform depth of the viscous high-percentage gels, a taped 11 × 14 cm gel tray was pre-incubated at 60 °C while the gel solution was prepared (~3 min). The gel was then poured into the pre-warmed tray in the incubator; a 10- or 12-well comb was inserted and the gel solution was allowed to settle for two minutes. The tray was then removed from the incubator and allowed to solidify at room temperature for at least 20 min. All gels contained 45, 50, or 55 mL total volumes and the solidified gels were approximately 6–8 mm thick. Gels were run in a submarine-style electrophoresis rig (Horizon 11–14 from Labrepco) in 0.5 × TB buffer at the indicated voltages and times. Most electrophoresis experiments were performed using a GE Healthcare EPS601 power supply. After electrophoresis, gels were stained for 40 min with mild shaking in 50 mL 2 × SYBR Gold (Invitrogen), which was created by diluting 10 μL SYBR Gold stock into 50 mL 0.5 × TB buffer (1:5000 dilution). To reduce background signal, the agarose gel was destained in ~150 mL 0.5 × TB for 15 min with shaking.

2.3. Polyacrylamide gel electrophoresis

1.5 mm thick 6.7% native polyacrylamide gels were prepared using 29:1 acrylamide:bis-acrylamide (National Diagnostics Accugel) in 1 × TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA). Gels were run in a vertical electrophoresis rig (Bio-Rad Mini PROTEAN Tetracell) at 200 V for 20 min at room temperature using either a BioRad PowerPac HC or Life Technologies PowerEase 300 W power supply. Each gel was stained for 10 min in 50 mL 2 × SYBR Gold as described above. No destaining was performed for the polyacrylamide gels.

2.4. Gel imaging and quantitation

Both agarose and PAGE gels were imaged using a Bio-Rad Molecular Imager ChemiDoc XRS + Imaging System set to the SYBR Gold Nucleic Acid Application with an exposure time of 0.6 s. Gels were quantitated with ImageLab (Bio-Rad, Inc.) using the externally-corrected, tight-binding method as previously described [33]. Band intensities were measured using the manual volume application within the program. All data used to calculate the $K_{D,app}$ employed the “adjusted volume” measures calculated by ImageLab, which are derived from the “local background” algorithm native to the program. The background signal from the agarose or the acrylamide gel was measured in an equal area of the gel that did not contain the binding samples. This background was subtracted from the sample volumes, and these background-corrected samples were plotted as the fraction of ligand bound versus protein concentration (Kaleidagraph, Synergy Software). $K_{D,app}$ values were calculated by fitting these data to the quadratic form of the binding isotherm