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 minutes) reduce opportunities for dissociation of bound complexes, an important concern in non-equilibrium nucleic acid binding experiments.

Keywords

RNA binding; DNA binding; binding affinity; nucleic acid binding protein; electrophoretic mobility shift assay; gel shift assay

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

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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 [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 hours 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 minutes, then allowing the solution to slow cool to room temperature over 30 minutes. Successful annealing was confirmed by agarose gel electrophoresis (Supp. Fig. 1A). The p19 siRNA binding protein (10 U/μL) 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 hour. 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 minutes). 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 minutes. 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 electrophoreseis experiments were performed using a GE Healthcare EPS601 power supply. After electrophoresis, gels were stained for 40 minutes 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 minutes 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 minutes at room temperature using either a BioRad PowerPac HC
or Life Technologies PowerEase 300W power supply. Each gel was stained for 10 minutes
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 seconds. 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

$$\frac{[PL]}{[L]_T} = \frac{(K_{D,app} + [P]_T + [L]_T) - \sqrt{(K_{D,app} + [P]_T + [L]_T)^2 - 4[P]_T[L]_T}}{2[L]_T}$$

(Eq. 1)

in which $[P]_T$ is the total protein concentration, $[L]_T$ is the total RNA ligand concentration,
[PL] is the concentration of protein-bound RNA, and $K_{D,app}$ is the apparent dissociation
constant.

3. Results

Previous work established that high percentage agarose gels (3 – 4%) run in 0.5× TB at high
voltages improved the resolution of dsDNAs between 20 and 150 bp as well as dsRNAs
between 17 and 150 bp [28,29]. These studies suggested that high-percentage, high-voltage
agarose electrophoresis is amenable to measuring the binding affinities of RNA/protein
interactions. However, a concern is that high voltages may force molecules to migrate
rapidly through the gel matrix, which may increase the risk of destroying the nucleic acid-
protein complexes [34]. Several parameters were evaluated in the current study to identify

the optimum conditions for separation and resolution of free RNA and protein-RNA complexes, using the p19/siRNA system as a model.

RNA was detected using SYBR Gold staining, a safe and sensitive detection method that is a widely-used alternative to the carcinogen ethidium bromide. Although 10 – 20 ng RNA could be readily detected (Supp. Fig. 1B), we observed that high concentrations of agarose caused a high level of background signal after SYBR Gold staining and destaining. To minimize background fluorescence caused by the retention of stain in the thick agarose matrix, we reduced both the concentration of agarose and the total volume of the gel (Fig. 1). Initial experiments that reduced the agarose concentration from 3.5% to 3.0% considerably reduced the background fluorescence (Fig. 1, panels A and B), even in a relatively large gel volume of 55 mL. Additional improvements in signal-to-noise were achieved by further reducing the agarose concentration to 2.5% while simultaneously reducing the total volume of the gel (and thus its thickness) from 55 mL to 45 mL (Fig. 1, panels C – E). The optimized conditions of 2.5% agarose and 45 mL gel volume maintained the excellent band resolution that was previously observed when running the gels at 20 – 25 V/cm. By minimizing the agarose concentration, we were also able to reduce the times needed to stain and destain the gel to 30 min and 15 min, respectively. In fact, longer incubations during staining and destaining were found to be counterproductive, because they allowed the RNA to diffuse in the gel, which significantly reduced both the resolution and signal-to-background ratio of the bands (Supp. Fig. 1C). From loading the sample to imaging the destained agarose gel, the total processing time using the optimized conditions was less than 1 hour.

Next, we identified the optimal voltage for separating the free and bound RNAs while retaining high resolution of both the free and bound species. Using a 3.0% agarose gel, we tested several voltages (35, 30, 25, and 20 V/cm, where cm refers to the total length of the gel) (Fig. 2). With decreasing voltage, we increased the run time from 6 min to 10 min to ensure that the high molecular weight protein/RNA complex entered the gel matrix. Higher voltages of 35 (data not shown) and 30 V/cm rapidly and cleanly resolved the free RNA. However, the protein-RNA complex was not stable at these voltages, as demonstrated by the presence of free RNA bands at higher protein concentrations as well as diffuse shifted RNA bands and increased smearing throughout the lane (Fig. 2, panels A–B). Reducing the voltage to 25 or 20 V/cm and running for 10 min allowed for clear separation of free and bound RNAs with excellent resolution of both species (Fig. 2, panel C).

The robust resolution that was achieved with the new high-percentage, high-voltage agarose gel conditions enabled quantification of the free and bound fractions of the RNA ligand, which can be used to determine the binding constants for the p19/RNA interaction. To directly compare the agarose system to a conventional polyacrylamide EMSA, we first optimized polyacrylamide EMSA conditions for the p19/RNA system. Briefly, 6.7% polyacrylamide minigels were run in $1\times$ TBE at 200 V for 20 min to separate free RNA from protein-bound RNA. Traditionally, polyacrylamide gels are often run in a cold environment to minimize heating from the electrophoretic field and promote protein stability [34]. However, in the p19 system, this approach was counterproductive, as the 2-nucleotide 3′ ssRNA overhang of the p19 dsRNA ligand has a T_M of 4 °C [35]. Therefore, the dsRNAs

were able to anneal and form multimeric complexes when the gels were run in the cold (Supp. Fig. 2). As observed in the agarose gel system, the RNA binding activity of p19 was stable at room temperature (Fig. 1 & 2). No significant heating was observed in the polyacrylamide gel system despite the high voltage (data not shown), which allowed the conventional native PAGE system to be run at room temperature as well.

Apparent binding constants were measured using the same reaction conditions followed by electrophoresis using both the agarose and the polyacrylamide gel systems in a head-to-head comparison. A range of p19 protein concentrations was incubated with 20 ng of dsRNA ligand, spanning well below and well above the published $K_{D,app}$ [31]. Half of each reaction was run on an agarose gel, while the other half was run on a polyacrylamide gel (Fig. 3, panels A and B). Three independent sets of agarose and polyacrylamide gel pairs were executed in this manner. After staining with SYBR Gold, the free and bound RNA bands were quantitated as described [33]. Because the dsRNA concentration in the binding reactions (~100 nM) was above the reported $K_{D,app}$ values for p19 in the mid-picomolar to low-nanomolar range [30,32], the data were fit with the quadratic form of the binding isotherm, which does not make any assumptions about the concentration of free protein relative to the concentration of total protein (Eq. 1).

To directly compare the ability of the native agarose gel shift to provide accurate measures of binding affinity, we carried out direct comparisons with a conventional native polyacrylamide gel. A set of binding reactions were incubated, and then divided between the high-voltage agarose gel and a conventional polyacrylamide gel for analysis (Fig. 3). Both systems yielded similar data quality and the fitted curves had similar shapes (Fig. 3, panels A, B, and C). The average $K_{d,app}$ of three independent replicates was determined. Using the standard polyacrylamide system, the $K_{d,app}$ was found to be 7.5 ± 1.4 nM, consistent with previous reports for the MBP-p19-CBD fusion protein [32]. Similarly, the new agarose system produced an average $K_{d,app}$ of 13.4 ± 1.3 nM. While the standard errors of the means do not overlap, the less than twofold difference in average $K_{d,app}$ values is within the generally accepted range of measurements in the field when using different methods.

4. Discussion

We have developed a native agarose gel system that enables rapid quantitation of RNA binding activity by proteins. We found that a 45 mL 2.5% agarose gel (approximately 7 mm thick) was ideal for optimal staining and destaining using SYBR Gold. Agarose gels are normally run in 1× TAE or 1× TBE buffer at ~10 V/cm to minimize artifacts caused by buildup of current and asymmetric heating effects [26,28]. Using a lower-conductivity buffer (0.5× TB), gels could be electrophoresed at 20 – 25 V/cm, which effectively and efficiently separated the unbound 21-nt dsRNA ligand (~12 kDa) from the RNA bound by the 40 kDa dimeric p19 protein in less than 10 minutes, with minimal smearing and good band resolution. When quantitated, this system produced measurements that were comparable to those obtained by conventional native polyacrylamide gel electrophoresis with minimal exposure to potentially toxic reagents. This represents a significant advance in electrophoresis methodology that is highly adaptable to a variety of protein-nucleic acid complexes, and permits the use of very short oligonucleotides.

One of the reasons that polyacrylamide gels have been used historically for EMSAs is the higher resolving power of polyacrylamide matrices, which allow for single-nucleotide resolution of oligonucleotides [10]. In contrast, agarose gels have a limited ability to resolve small oligonucleotides, especially those that differ in size by only a few nucleotides [10]. Several different approaches have been taken in an effort to improve agarose gels for use in EMSAs with shorter DNAs. Increasing the percentage of agarose to 1 – 1.5% in one study improved band resolution, but run times were still fairly long which allowed for diffusion of the DNA [24]. The innate resolving power of an agarose matrix has also been improved by manipulating the gel composition through the addition of other chemicals, such as metals and amines [26] or polysaccharides [25,36]. Still other approaches employed chemically-modified agarose, in the form of hydroxyethylated agarose molecules, in an effort to reduce pore size and thereby improve resolution [22,27].

However, the most promising and widely-applicable alteration to the agarose system involves simply altering the running buffer [23]. In particular, it was recently demonstrated that reducing the conductivity of the running buffer could improve the resolving potential of an agarose gel by allowing for the use of higher voltages and significantly shorter gel run times [28]. The work described here has continued to develop the applications of these high-voltage agarose gel systems. Gel shift assays like EMSAs substantially benefit from the potential for shorter gel run times, which minimizes the time during which irreversible dissociation of the protein/ligand complex can occur. This allows for a more accurate quantitation of the initial equilibrium binding states, thereby yielding robust measurements of the apparent equilibrium dissociation constant ($K_{D,app}$).

The adaptation of the agarose gel system for protein/RNA EMSAs benefitted from optimizing several parameters, including agarose content, gel thickness, and voltage. We reduced the fluorescence background produced after staining with SYBR Gold by minimizing agarose concentration and gel thickness, as well as optimizing the destaining protocol by incubating in TB instead of water. These gel parameters can be adjusted for the protein:RNA system of interest, as well as each individual lab's gel and detection systems of choice. Possible detection methods that are more sensitive than SYBR Gold staining, such as fluorescent labels, radiolabels, and biotinylation, may be considered [3,10,17]. An additional advantage to optimizing the agarose concentration of the gels along with screening multiple voltages was to improve band resolution and band signal intensity, while simultaneously reducing the amount of smearing between the shifted and unshifted ligand bands. Therefore, particular attention should be paid to these parameters when establishing this method for an individual protein:RNA system, as the unique binding affinities and binding rates of each system will likely require individual optimization.

The commercially-produced recombinant p19 protein employed in this study was previously reported to have low nanomolar affinity for miRNAs [32] (NEB Technical Support, personal communication). A direct comparison between the new agarose system and a conventional polyacrylamide system was carried out by analyzing the same binding reactions in both systems. We determined that the two methods produced similar measurements of binding affinity. There was less than a twofold difference in the measured $K_{D,app}$ values using the two methods (13.4 ± 1.3 nM using agarose versus 7.5 ± 1.4 nM with polyacrylamide). As

noted above, these values are consistent with published data for the MBP-p19-CBD fusion protein, and demonstrate that the new agarose-based system is able to quantitatively measure high-affinity and physiologically relevant interactions between proteins and RNAs.

4.1 Conclusions

In summary, we have developed an electrophoretic mobility shift assay method using an agarose gel and low-conductivity buffer system that requires only standard molecular biology reagents and equipment. The assay allows for rapid, quantitative analysis of protein/RNA interactions, requiring less than 90 minutes from gel pouring to data acquisition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used

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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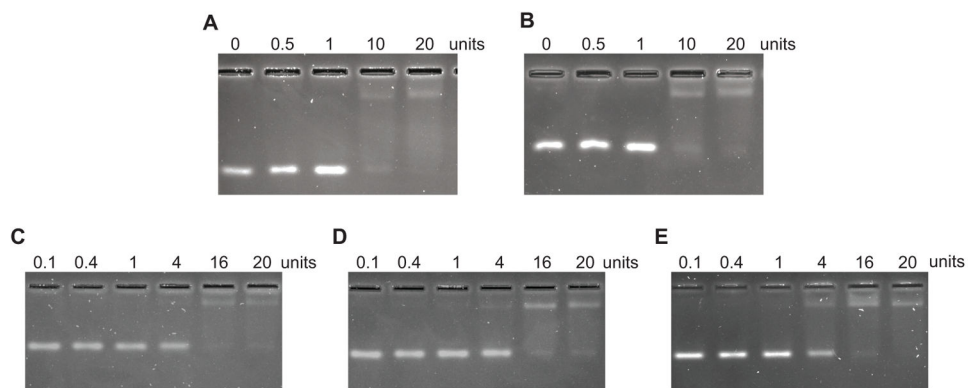
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Figure 1. Reduction of the fluorescent background improved the signal-to-noise ratio
Agarose concentration and gel thickness were optimized to reduce the amount of fluorescence retained by the agarose gels. *(A and B)* 55 mL agarose gels were run at 25 V/cm for 10 min. Protein concentrations ranged from 0 to 1 U/µL. *(C, D, and E)* Agarose gels were run at 20 V/cm for 10 minutes at room temperature. Protein concentrations ranged from 0 to 0.8 U/µL. Gels were stained in SYBR Gold for 30 minutes and destained in 0.5× TB for 15 minutes before imaging.
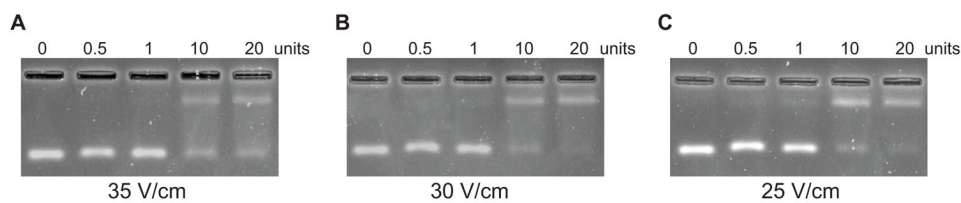
Figure 2. Optimization of voltage improves the resolution of both free and protein-bound RNA species

30 ng of dsRNA was incubated with increasing concentrations of p19 protein (0 – 1 U/µL) and then the bound and free complexes were separated on 2.5% agarose gels at varying voltages at room temperature. *(A)* 30 V/cm; *(B)* 25 V/cm; and *(C)* 20 V/cm. Gels were stained in SYBR Gold for 30 minutes and destained in 0.5× TB for 15 minutes before imaging.
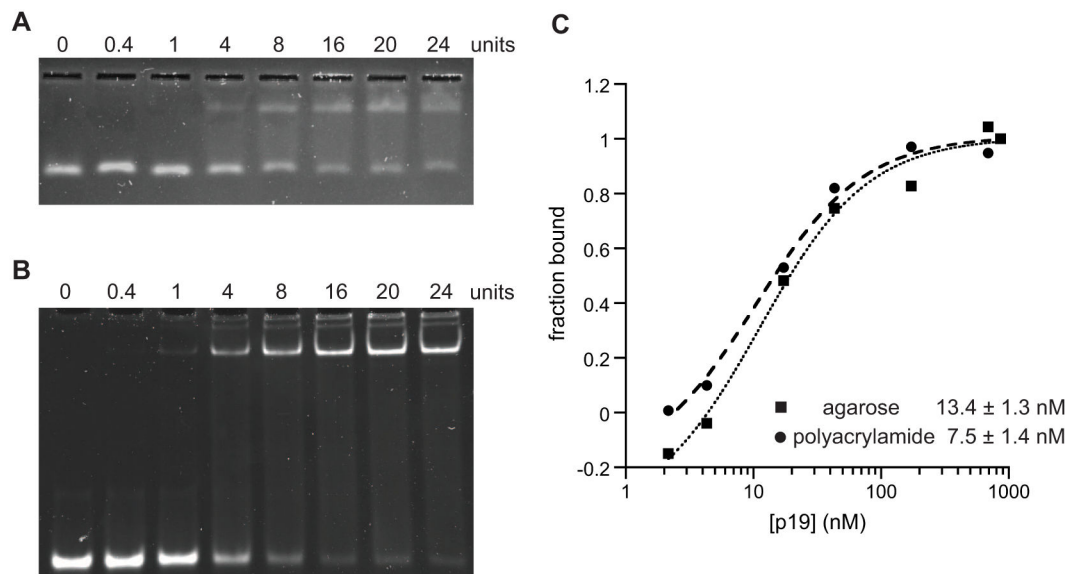
Figure 3. High-voltage, high-percentage agarose gels yield $K_{D,app}$ values comparable to conventional PAGE

Representative EMSA data using either *(A)* an agarose or *(B)* a polyacrylamide gel to separate bound and free RNA ligand. *(C)* The gels shown in *(A)* and *(B)* were quantitated and subjected to multivariate fitting using the Langmuir binding isotherm to determine the $K_{D,app}$. *(D)* The average $K_{D,app}$ values (± SEM) for three independent replicate experiments analyzed by the two gel systems.