Published in final edited form as:

Electrophoresis. 2010 January ; 31(4): 648–653. doi:10.1002/elps.200900573.

Stabilizing labile DNA-protein complexes in polyacrylamide gels

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Abstract

The electrophoretic mobility shift assay (EMSA) is one of the most popular tools in molecular biology for measuring DNA-protein interactions. EMSA, as standardly practiced today, works well for complexes with association binding constants $K_d > 10^9 \text{ M}^{-1}$ under normal conditions of salt and pH. Many DNA-protein complexes are not stable enough so that they dissociate while moving through the gel matrix giving smeared bands that are difficult to quantitate reliably. In this work we demonstrate that the addition of the osmolyte triethylene glycol to polyacrylamide gels dramatically stabilizes labile restriction endonuclease EcoRI complexes with nonspecific DNA sequences enabling quantitation of binding using the electrophoretic mobility shift assay. The significant improvement of the technique resulting from the addition of osmolytes to the gel matrix greatly extends the range of binding constants of protein-DNA complexes that can be investigated using this widely used assay. Extension of this approach to other techniques used for separating bound and free components such as gel chromatography and capillary electrophoresis is straightforward.

Keywords

DNA-protein complex; EMSA; neutral osmolytes; stabilization; triethylene glycol

1 Introduction

The electrophoretic mobility shift assay (EMSA)[1, 2] is a standard and extremely popular tool in molecular biology for measuring DNA-protein complex formation. The technique uses polyacrylamide gel electrophoresis to separate DNA-protein or RNA-protein complexes from free DNA or RNA. It has been applied routinely for last 30 years to study protein-nucleic acids interactions [3–7]. EMSA has been extensively applied to the investigation of the binding of transcription factors and other gene regulatory proteins to DNA and proved quite useful for identifying unknown proteins that bind specifically to the promoter and enhancer regions. Parsing the intricacies and interconnections of gene regulation depends critically on the technique. Additionally, it has been also used to study DNA binding of repair proteins, polymerases, and restriction endonucleases. EMSA is a useful technique for probing the effects of drug-DNA adducts on the regulatory proteins binding to these modified DNA regions in order to assess the ability of the drugs to disrupt gene regulation associated with disease or abnormal cell function. A literature search using Scopus reveals that almost 8000 articles and 160 review articles containing the term ‘gel mobility shift assay’ in the title, abstract, or key words, have appeared in the biomedical literature since 2000. These impressive numbers are not surprising considering numerous

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The authors have declared no conflict of interest.

advantages of the EMSA compared with other techniques. EMSA is extremely sensitive; picogram quantities of radioactively labeled DNA will suffice, consequently saving oftentimes limited materials. Association binding constants as high as 10^{11} – 10^{12} M⁻¹ can be conveniently measured. At the same time EMSA provides vast flexibility both in sample volume and sample concentration. Additionally, it is easy to adjust the acrylamide percentage to optimize conditions for separating complexes of very different molecular weights. Lastly, the EMSA technique is simple and easily accessible to researchers (see [8] and references cited there for the latest EMSA developments and protocols).

Polyacrylamide gels stabilize DNA-protein, RNA-protein, or protein-protein complexes by a crowding or caging mechanism. Even if complexes should dissociate in the gel, there is a reasonable probability to re-associate before the components can separate because of the constraining gel network. Still, every technique has its limitations. EMSA, as standardly practiced today, works well for complexes with association binding constants $K_a > 10^9$ M⁻¹ under normal conditions of salt and pH. Many nonspecific DNA-protein complexes and some specific complexes as well, however, are not stable enough, so that they do dissociate while moving through the gel matrix giving smeared bands that are difficult if not impossible to quantitate precisely.

In order to extend the applicability of the EMSA to these labile complexes, we have investigated the effect of adding stabilizing osmolytes (such as glycerol or triethylene glycol) to the gel itself. This is a logical extension of our previous work on the osmolyte concentration sensitivity of the equilibrium constant for the binding reaction of free protein to its recognition DNA sequence [9, 10], of the relative equilibrium constant for the competitive binding of protein to specific site and nonspecific DNA [9–11], and of the dissociation rate of specific site complexes [12–14]. In particular, we have shown previously that neutral osmolytes can strongly decrease the rate of DNA-protein complex dissociation. For example, the half-life time of the restriction endonuclease EcoRI complex with its specific DNA sequence increases about 16 fold from 0 to 1 osmolar triethylene glycol [13]. This finding led us to develop a method that uses osmotic stress to ‘freeze’ mixtures of DNA-protein complexes and prevent further reaction enabling analysis of the products [15]. We are now extending this approach adding high enough concentration of neutral solutes to the gel matrix to stabilize weak DNA-protein complexes as they move through the gel.

The dissociation of complex in a gel necessarily exposes the protein and DNA surface area that was buried in the complex. The stabilizing effect of osmolytes on protein-DNA complexes can be rationalized by the exclusion of osmolytes from exposed protein and DNA surfaces. This exclusion is due to both solute size (a crowding effect) and to a preference of both DNA and proteins for water rather than for solutes. Solute, protein, and DNA surface hydration energies are generally much more favorable than the direct interactions of solute with protein or with DNA. This osmolyte exclusion from protein and DNA surfaces necessarily means a water inclusion. The energetic consequences of solute exclusion can be calculated as an osmotic work. If the volume of included water on the surface of protein and DNA is V_w and the osmotic pressure of the solute excluded from this water is Π_s , then the energy for the exclusion of osmolyte from protein or DNA is $\Pi_s \cdot V_w$. The extent of exclusion, V_w , depends sensitively on the nature of the osmolyte, protein, and DNA through the osmolyte-protein and osmolyte-DNA interaction energies. We have found that triethylene glycol is particularly strongly excluded from protein and DNA surfaces in comparison to the other solutes we have investigated, glycine betaine, trimethylamine oxide, glycerol, α -methyl glucoside, sucrose, stachyose, t-butanol, sorbitol, and ethylene glycol. Additionally, triethylene glycol is not particularly viscous and does not slow electrophoretic velocities as much as sucrose, for example, when used at the same concentrations.

As a proof of principle, we have focused here on using triethylene glycol and glycerol in polyacrylamide gels to stabilize complexes of the restriction endonuclease EcoRI with nonspecific and a 'star' sequence oligonucleotide that we studied previously. 'Star' sequences are sequences that differ from the recognition sequence, GAATTC, by a single base pair and are cleaved by the nuclease at very low frequency [16]. EcoRI binds $\sim 1-2 \times 10^4$ – fold weaker to nonspecific oligonucleotides than to the specific recognition site [11, 17, 18]. The enzyme binds only about $\sim 2-6$ fold stronger to the TAATTC 'star' sequence than to nonspecific sequences [17, 18].

2 Material and methods

2.1 Materials

Triethylene glycol was purchased from Fluka Chemical Corp. and glycerol from Invitrogen. The sequences of the double-stranded 24 and 30 bp long oligonucleotides used in the binding experiments were:

Specific sequence GAATTC oligo: GGCGATCGAGA**ATTCT**CGATCGCC

TAATTC sequence oligo: ACGACGGCCAGT**TAATTC**GAGCTCGGTACC

CTTAAG sequence oligo: ACGACGGCCAGT**CTTAAG**GAGCTCGGTACC.

The specific sequence oligonucleotide contains the EcoRI cognate recognition site, GAATTC (in bold). The TAATTC oligonucleotide contains a first base pair substitution of the recognition sequence and is commonly termed a 'star' site. In the nonspecific oligonucleotide, the 'star' sequence is replaced by an inverted specific sequence (CTTAAG) or a nonspecific site with all six base pairs wrong. The oligonucleotides purchased from Invitrogen were hybridized and purified as described previously [18]. Highly purified EcoRI was a gift from Dr. L. Jen-Jacobson.

2.2 Complex preparation

The EcoRI-DNA binding buffer typically included 20 mM ImidazoleCl (pH 7.0), 20% (v/v) triethylene glycol, 2 mM DTT, 1 mM EDTA, and 0.02% NP-40. We have not encountered any problems when NP-40 or BSA was added to the reaction mixture, but caution should be exercised adding double-stranded nonspecific DNA competitors. It is important to choose competitor that would bind to the protein much weaker than the nonspecific sequence of interest. Obviously, binding of any nonspecific DNA sequence to the protein will be increased in the presence of neutral solutes to a similar extent as the binding of the 'star' and nonspecific oligonucleotides of interest.

The salt concentration was 50 mM NaCl for the 'star' and nonspecific sequence complexes and 80 mM NaCl for the specific site oligonucleotide. The conditions were chosen based on our previous work [13, 18] to give stoichiometric binding of protein to the specific, 'star' and nonspecific sequence DNA oligonucleotides. EcoRI binds stoichiometrically to the specific sequence at 90 mM NaCl and pH 7.0 even without osmolyte present. Specific equilibrium binding constant at these conditions can be estimated as at least $\sim 5 \times 10^9 - 10^{10} \text{ M}^{-1}$ [11, 13]. The nonspecific binding constant is about 10^4 times smaller. Decreasing the salt concentration to 50 mM NaCl and increasing the triethylene glycol concentration to 20% (~ 2 osm) stabilizes nonspecific and non-cognate binding by at least 2000 fold [13, 19] bringing nonspecific equilibrium association constant to $\sim 1 \times 10^9 \text{ M}^{-1}$ at minimum. The concentrations of protein and DNA were ~ 80 nM EcoRI and 160 nM oligonucleotide. If complexes are preserved during electrophoresis we expect to measure fraction of bound oligonucleotides equal to 0.5 for all three oligonucleotides used.

2.3 Gel mobility-shift experiments

The reaction mixtures of EcoRI and oligonucleotides were electrophoresed in 10% polyacrylamide mini-gels (acrylamide:bis-acrylamide=29:1), 1xTAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) with or without added triethylene glycol or glycerol. Samples were loaded on the gel at 150–200 V, and run for ~1 hour. The osmolytes triethylene glycol and glycerol were added to the acrylamide gel mix before polymerization was initiated by ammonium persulfate and TEMED. These solutes do not interfere with the polymerization reaction. The pH of the TAE buffer decreased from 8.25 with no added osmolyte to 8.05 with 30% triethylene glycol. To minimize loss of solute in the gel by diffusion, 40 μ l of 1xTAE mixed with triethylene glycol or glycerol were also added to the sample wells as soon as gel was immersed in the electrophoresis buffer. The concentration of osmolyte in the electrophoretic well matched the concentration of the solute in the gel matrix. To ensure that density of the loaded samples would exceed density of the buffer loaded in the well, either 5.3% (triethylene glycol gels) or 8% (glycerol gels) ficoll (70K MW) was also in the reaction mix. We found that adding osmolytes to the running buffer did not additionally improve stability of the complexes in the gel.

Electrophoretic bands containing free DNA and DNA-protein complex were stained with the fluorescent dye SYBR Green I (Invitrogen) for 30–40 minutes. Longer staining times led to a decrease in the intensity probably due to loss of the oligonucleotide from the gel. The gels were imaged with a FLA-3000 Fluorescent Image Analyzer (Fuji Film). The FLA-3000 was interfaced to a Pentium PC. Band intensities were quantified using the Fuji Film software MultiGauge for Windows. The linearity of oligonucleotides fluorescent staining was confirmed over the range of DNA concentrations studied.

3 Results and discussion

Figure 1a shows a 10% polyacrylamide gel in standard TAE buffer with no osmolyte added. As is our standard practice with DNA-protein complexes, all samples contained triethylene glycol (20% v/v) to prevent complex dissociation before entering the gel [13–15]. The free and protein-bound oligonucleotides are fluorescently stained with SYBR Green I. The ‘star’ and nonspecific sequence oligonucleotides are 30 bp long while the specific site oligonucleotide is only 24 bp, accounting for the difference in migration seen in the gel. The specific complex is quite stable in the gel. The complexes of protein with nonspecific and ‘star’ sequence oligonucleotides simply give a smear in the gel. The fluorescence intensity profiles along the lane for the specific and ‘star’ sequence oligonucleotides are shown in Figures 1b and 1c. The profile of the specific sequence complex is typical of a stable, non-dissociating complex; the free and complex DNA bands are separated by a flat baseline. The fraction of oligonucleotide in the specific sequence complex is ~0.5 as expected for stoichiometric binding and from the ratio of protein to DNA in the reaction mix. Most of the ‘star’ sequence complex, however, dissociates almost immediately upon entering the gel. The complex continues dissociating throughout the gel as seen by the decreasing background intensity between the free DNA band and the presumed position of the complex. The small blip indicated by the arrow in Figure 1c is all that is left of the complex at the end of the electrophoresis run. The lane profile does not depend on the osmolyte concentration of the sample in the well (data not shown).

Figures 2a–c show gels with triethylene glycol added to the gel matrix. As in Figure 1a, each three lane gel shows the separation of complex and free DNA for EcoRI mixtures with the specific sequence, ‘star’ sequence, and nonspecific sequence oligonucleotides. Under the stoichiometric binding conditions of the reaction mix, about equal fractions of DNA are expected in the complex and free oligonucleotide bands. The concentration of triethylene glycol in the gel is 10, 20, and 30 % (v/v) for a, b, and c, respectively. As is readily apparent,

both the nonspecific and 'star' sequence complexes become considerably more stable in the gel as the concentration of triethylene glycol is increased.

Lane intensity profiles are shown in Figures 2d–f for the 'star' sequence complex as in Figure 1c. At both 10 and 20 % triethylene glycol, some complex dissociation is apparent as seen by the sloping baseline between the free DNA and complex bands and by the smaller than expected fraction of complex. At 30% triethylene glycol, however, essentially no difference in the profiles is seen among the specific, 'star' sequence, and nonspecific (data not shown) complexes. The fraction DNA in the complex bands is ~ 0.5 for all three oligonucleotides confirming that even nonspecific complexes are stable enough in the gel matrix to be analyzed reliably by EMSA.

Glycerol has been added to polyacrylamide gels to stabilize nucleosome isoforms [20] but never to our knowledge to stabilize weak protein-DNA complexes. Glycerol, however, is not an optimal solute; it is not as strongly excluded from DNA and protein surfaces as is triethylene glycol. Figures 3a–b show gel mobility shifts assays for specific, 'star' sequence, and nonspecific EcoRI complexes using 10% polyacrylamide gels with added 30% glycerol. 'Star' sequence complex dissociation is readily apparent. The fraction of total DNA in the 'star' sequence complex band is only half that of the specific recognition site. 30% glycerol is comparable to ~15 % triethylene glycol (data not shown).

4 Concluding remarks

The results clearly demonstrate that including the osmolyte triethylene glycol in the gel can dramatically stabilize the weak non-cognate and nonspecific complexes of EcoRI for analysis. Without solute added to the gel matrix, both 'star' and nonspecific sequence complexes simply dissociate too quickly in 10% polyacrylamide gels. We showed here that 30% triethylene glycol in the gel (equivalent to ~ 3.7 osmolal) is enough to stabilize complexes that have association binding constants in the range of 10^5 – 10^6 M⁻¹ at physiological salt and pH conditions. The purpose of this paper is to demonstrate that weak complexes can be made stable enough to survive gel electrophoresis. We, of course, do not intend that initial binding reactions must necessarily contain a very large concentration of osmolyte. In an actual binding experiment, the initial binding reaction would occur under regular conditions of salt, pH, etc. Our practice is to then "freeze" the binding distribution at equilibrium by adding enough specific oligonucleotide to bind free protein and enough osmolyte to greatly slow complex dissociation in the electrophoretic well [15] and now to stabilize the complex in the gel. Under such conditions, the distribution of bound and free DNA of interest in the gel represents the equilibrium before the stop reaction mixture was added, and the equilibrium binding constant can be measured reliably.

Of course, nonspecific complexes of the EcoRI were chosen just as an example to illustrate the principle. There is no reason why even less stable DNA-protein complexes with association constants in the 10^3 – 10^4 M⁻¹ range can not be made stable enough for the gel mobility shift assay considering that neutral osmolytes can be added to very high concentrations without apparently affecting the integrity of DNA-protein complexes. The technique can be readily used for any RNA and DNA-protein complexes as well as protein-protein complexes that are sensitive to the osmotic stress. In general, disruption of the complex is accompanied by an uptake of water from the bulk solution required for the hydration of surfaces that were "hidden" from the solvent in the complex. This process is energetically unfavorable at the conditions of low water activity (high concentrations of osmolytes). Indeed, the stabilization effect of osmolytes on DNA-protein complex has been demonstrated already for several DNA regulatory proteins: *Escherichia coli gal* [9], *lac* [21], *tyr* [22], and Cro [14] repressors, *E. coli* CAP protein [23], the restriction

endonucleases EcoRI [11, 13, 18, 24], and BamHI [10], and the TATA-binding protein [25]. We also saw stabilization effect of osmolytes on the EcoRV restriction endonuclease complex with DNA and on the repair protein *Taq* MutS complex with its recognition sequence (our unpublished results).

Extension of this approach to other techniques for separating bound and free components such as gel chromatography and capillary electrophoresis is straightforward.

Abbreviations

EMSA electrophoretic mobility shift assay

Acknowledgments

We are deeply grateful to V.A. Parsegian for the valuable discussion and support and to L. Jen-Jacobson for the kind gift of highly purified EcoRI.

Funding

This work was supported by the Intramural Research Program of the National Institutes of Health, NICHD (Eunice Kennedy Shriver National Institute of Child Health and Human Development).

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1 bc

Figure 1.

a) The separation of free DNA and DNA-protein complex in a 10% polyacrylamide gel in 1xTAE without added osmolyte is shown for mixtures of EcoRI with oligonucleotides containing the specific recognition sequence of EcoRI, a noncognate 'star' sequence, and a completely nonspecific sequence. The upper band corresponds to DNA in complex and the lower to free DNA. The conditions used in this experiment were: 20 mM Imidazole pH 7.0, 20% (v/v) triethylene glycol, 50 mM NaCl for the nonspecific and 'star' sequence oligonucleotides and 80 mM NaCl for the specific sequence oligonucleotide.

b) The summed fluorescent intensity along the specific sequence oligonucleotide lane is shown. The complex band is typical of a stable DNA-protein complex.

c) The summed fluorescent intensity along the 'star' sequence oligonucleotide lane is shown. It is apparent from the fluorescent intensity between the complex and free DNA bands that the complex dissociates in the gel. Initially the amount of complex was about the same as in (b). The arrow indicates the small fraction of complex that survived electrophoresis.

2d-f

Figure 2.

(a–c). The separation of free DNA and DNA-protein complex in a 10% polyacrylamide gels in 1xTAE with 10% (a), 20% (b) or 30% (c) triethylene glycol added to the gel is shown for mixtures of EcoRI with oligonucleotides containing the specific recognition sequence (Sp) of EcoRI, a non-cognate ‘star’ sequence (*), and a completely nonspecific sequence (Nsp) as in Figure 1a. The marked stabilization of EcoRI complexes with ‘star’ and nonspecific sequence oligonucleotides is readily apparent.

After gels were immersed in the electrophoretic chamber, 40 μ l of 1xTAE buffer containing 10% (a), 20% (b) or 30% (c) of the triethylene glycol were added to each well to minimize

loss of the triethylene glycol from the gel by diffusion. The sample buffer conditions used in this experiment were as described in the legend to Figure 1a.

d) The lane intensity profile is shown for the 'star' sequence oligonucleotide with 10% triethylene glycol added to the gel. Substantial dissociation of the complex can be inferred from the intensity smear on the left-hand side of the free DNA band. The height of the complex peak is much smaller than the free DNA peak.

e) The lane profile is shown for the 'star' sequence oligonucleotide with 20% triethylene glycol added to the gel. Much less dissociation of the complex occurs in 20% osmolyte than 10%, but some dissociation is still apparent.

f) The lane profile is shown for the 'star' sequence oligonucleotide with 30% triethylene glycol added to the gel. The profile is virtually indistinguishable from the specific sequence profile shown in Figure 1b. From a practical viewpoint, the 'star' sequence complex is stable enough for the electrophoretic mobility shift assay.

3b

Figure 3.

a) The separation of free DNA and DNA-protein complex in a 10% polyacrylamide gel in 1xTAE with 30% glycerol added to the gel is shown for mixtures of EcoRI with oligonucleotides containing the specific recognition sequence (Sp) of EcoRI, a noncognate 'star' sequence (*), and a completely nonspecific sequence (Nsp) as in Figure 1a. After gels were immersed in the electrophoretic chamber, 40 μ l of 1xTAE buffer containing 30% of the glycerol were added to each well to prevent loss of glycerol in the gel by diffusion. The sample buffer conditions used in this experiment were as described in the legend to Figure 1a.

b) The lane profile is shown for the 'star' sequence oligonucleotide with 30% glycerol added to the gel. Dissociation of the complex is apparent from the sloping baseline intensity leading to the free DNA band and from the relative amplitudes of the complex and free DNA bands. 30% glycerol has the same effectiveness on complex stability as ~15% triethylene glycol.