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Journal of Chromatography B, 789 (2003) 431–435
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Capillary electrophoresis-based method to quantitate DNA–protein interactions

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Received 9 December 2002; received in revised form 17 January 2003; accepted 20 January 2003

Abstract

A novel, rapid and simple capillary electrophoretic mobility shift assay (CEMSA) with laser-induced fluorescence (LIF) has been developed for the quantitative study of protein–DNA interactions. This method is particularly useful for the study of basic proteins, the most common of the DNA-interacting proteins. To avoid protein stickiness to the capillary walls we have introduced the use of neutral polyacrylamide that requires the use of reverse polarity. Under these conditions, excellent separation of DNA and protein–DNA complexes was obtained without the requirement of a gel matrix, thereby allowing the easy and reliable quantification of protein–DNA affinities. Analysis of the affinities of histones H2B and H4 for a synthetic oligo have been used to demonstrate the reproducibility and accuracy of this method. We have observed that H4 has a higher affinity for DNA than H2B, with half saturation fractions lying in the micromolar range.

Keywords: DNA; Proteins; Histone

1. Introduction

DNA–protein interaction is a critical issue in a wide range of biological studies, such of those on chromatin structure and regulation of gene function. There are several established techniques for protein–DNA binding studies, among which electrophoretic mobility shift assays (EMSA) [1] are of particular importance. Although useful in many different protein–DNA binding studies, EMSA are not optimal for quantitative analysis, and the obtaining of accurate affinity constants by this method is a tedious task [1]. In contrast, the calculation of protein–DNA affinities can be precisely obtained by calorimetric experiments [2] but the required equipment is not usually available in biochemistry and molecular biology laboratories.

Alternatively, high-performance capillary electrophoresis provides the possibility of developing accurate and reproducible methods to quantitate protein–DNA affinities based on its ability to separate at high-resolution macromolecular complexes from their isolated interacting subunits.

In fact, the quantification of ligand–DNA interactions by high-performance capillary electrophoresis

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1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00103-X

on untreated fused-silica capillaries has been widely reported (reviewed in Ref. [3]), including that of protein with double and single-stranded DNA [4–9]. Generally, DNA-interacting proteins are rich in basic amino acids, which cause adsorption to surfaces. This is in fact, the major drawback of the previously described methods where adsorption by the charged sites of proteins on fixed, negatively charged sites on the capillary wall occurs. This process leads to band-broadening and, it results in far lower actual plate numbers than expected [10] and complicates the obtaining of accurate affinity constants.

Here we describe a novel capillary electrophoretic mobility shift assay (CEMSA) that exploits a neutral coating capillary (Beckman Coulter, Madrid, Spain) to avoid protein adsorption onto the capillary walls. The internal silica capillary walls of neutral capillaries are covered with a neutral polyacrylamide to deactivate the silanol groups, thereby reducing the electrostatic interactions between samples and the capillary wall. Under these conditions, electrosmotic flow is virtually absent, and so the system requires reverse polarity (anode at the detector end).

Histones were chosen to develop the method considering their basic charge and their well-characterized binding properties (reviewed in Ref. [11]). In this regard, histones have also been previously used as a model for the development of new separation techniques of nuclear DNA-interacting proteins [12].

2. Experimental

A neutral coating capillary (Beckman Coulter) (32.5 cm×50 μm, effective length 20 cm) was used in a P/ACE MDQ capillary electrophoresis system (Beckman Coulter) connected to a Karat Software® data-processing station. The running buffer (40 m*M* Tris–borate, 0.95 m*M* EDTA, pH 8.0) was found to yield a better peak shape and resolution than did citrate–MES, pH 6.0, Tris, pH 8.0, and MOPS, pH 7.0, which were also tested during the development of the method. The former buffer produces low current when working at high voltage (30 kV, 923 V/cm), thus favoring the stability of protein–DNA complexes during separation. Different running temperatures between 15 and 25 °C and operating voltages between 20 and 30 kV were also tested. The best separation of peaks for the shortest time period was obtained with 20 °C running temperature and using a constant voltage of 30 kV. Laser-induced fluorescence (LIF) was detected by excitation at 488 nm (3-mW argon ion laser provided by Beckman Coulter) and emission collected through a 520-nm emission filter (Beckman Coulter). Samples were injected under pressure (0.2 p.s.i.) for 3 s and the running temperature was maintained at 20 °C. Before each run, the capillary was conditioned by washing with running buffer for 2 min. Buffers and running solutions were filtered through 0.2-μm pore-size filters.

Binding experiments between histones H4 and H2B and a double-stranded standard synthetic oligo (dsSSO) were conducted in order to evaluate the method. SSO sequence was chosen at random since core histones have not specific DNA targets. Histones H2B and H4 were purified as previously described by Ballestar et al. [13] and chicken erythrocyte nuclei were obtained as described by Weintraub et al. [14]. Individual histone fractions were prepared from chicken erythrocyte nuclei by acid extraction followed by acetone precipitation, and they were further purified by reversed-phase HPLC on a Delta-Pak C_{18} column (Waters Cromatografía, Barcelona, Spain) eluted with an acetonitrile gradient (20–60%) in 0.3% trifluoroacetic acid. Fractions were freeze-dried and routinely checked by SDS–PAGE on 15% gels [15]. Pure histone fractions were pooled. DNAs, purchased as single-stranded oligonucleotides (Qiagen, Grawley, UK), were as follows: Forward SSO (GATCCGACGACGACGACGACGACGACGACGACGACGATC) and reverse SSO (GATCGTCGTCGTCGTCGTCGTCGTCGTCGTCGT CGGATC). Forward oligonucleotides were labelled at their 5′ ends with 6-FAM. Complementary oligonucleotides were mixed at equimolar concentrations, and annealed by bringing the solution to 95 °C and allowing it to cool down slowly to room temperature. Binding reactions were performed in binding buffer as described [16]. Increasing amounts of histone H2B and H4 were added to 6-FAM-labelled DNAs in binding buffer (10 m*M* Tris–HCl, pH 8.0, 3 m*M* $MgCl_2$, 50 m*M* NaCl, 0.1 m*M* EDTA, 0.1% NP-40, 2 m*M* DTT, 5%

glycerol and 0.4 mg/ml BSA) and incubated at 37 °C for 15 min.

The saturation of the dsSSO for H4 and H2B was quantified using GraFit 3.1 software (Erithacus software, Horley, UK). In brief, the saturation of the oligo (R=(complex peak area)/(complex peak area+free oligo peak area)) was plotted against increasing quantities of histones. The concentration required for 50% saturation of binding ($R_{1/2}$) was then calculated, seeking the best fit of the data to different binding models/curves.

3. Results and discussion

Excellent resolution of free DNA and DNA–protein complex was obtained under the described conditions (Fig. 1A) with no gel matrix required, thus avoiding putative undesirable effects of the sieving gel on the binding affinity [17]. This represents a reliable advantage over other capillary gel electrophoretic methods for protein–DNA binding assays that engage protein absorption to the capillary walls. Under these conditions, the migration time of the oligo is around 4.3 min while for the complex it is around 5.3 min. Thus, including the rinsing step, each analysis takes less than 8 min. The appearance and subsequent increase of the protein–DNA complex was concomitant with the decrease in the area of the peak corresponding to the free probe. The high resolution of this technique indicates that a single retardation peak corresponds to the binding of a single molecule of protein to the DNA probe. Bovine serum albumin, used as a negative control, did not produce any retardation peak (not shown). Triplicates were obtained for each histone concentration.

The excellent reproducibility of the saturation of the oligo (R) calculated by this method is shown in

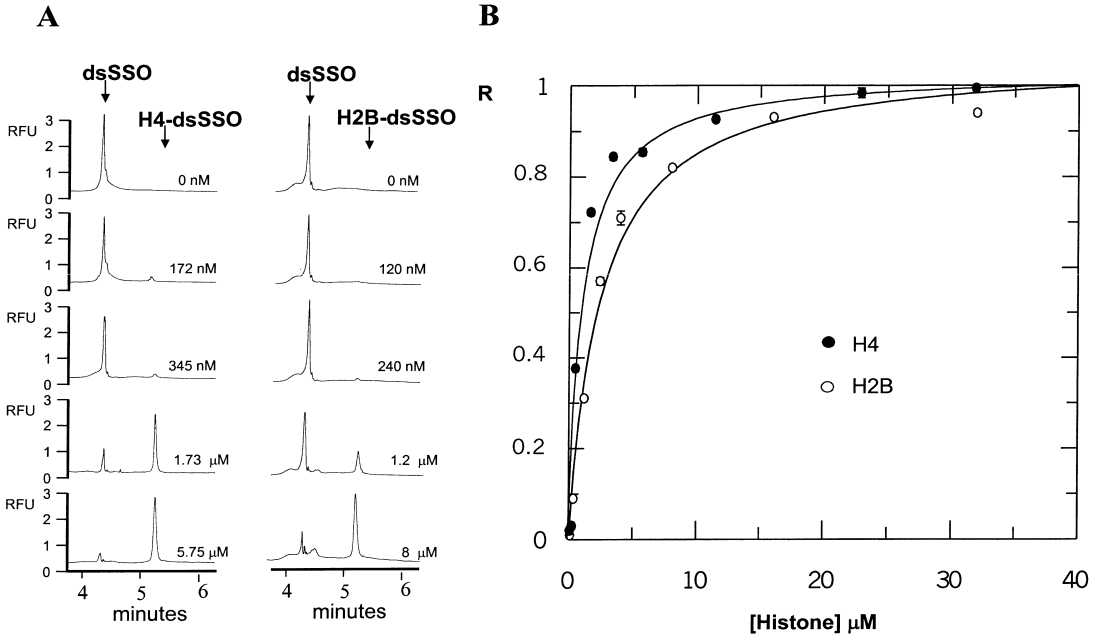

Fig. 1. Determination of histone–DNA binding by CEMSA. (A) Electropherograms for mixtures of the double-stranded oligo (dsSSO) (24 nM) and increasing concentrations of histone H4 (left panel) and histone H2B (right panel) in 10 mM Tris–HCl, pH 8.0, 3 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, 0.1% NP-40, 2 mM DTT, 5% glycerol and 0.4 mg/ml BSA. Analytical conditions: 32.5 cm×50 μm capillary (effective length 20 cm); low pressure injection at 0.2 p.s.i. for 2 s; 20 °C; 30 kV voltage; reverse polarity (anode at the detector end); buffer, 40 mM Tris–borate, 0.95 mM EDTA, pH 8.0; laser-induced fluorescence detection: excitation at 488 nm, emission at 532 nm. RFU, relative fluorescence units. (B) Binding fit for histone H4 (black circles) and histone H2B with dsSSO using GraFit 3.1 software. R, saturation of the oligo ([complex]/[complex]+[DNA]). Assuming single-site binding, $R_{1/2}$ was calculated to be 1.09±0.3 μM for H4 and 2.43±0.4 μM for H2B. Results are expressed as the mean of three replicates±SD.

Table 1
Reproducibility of the method with constant amounts of the dsSSO oligo (24 n*M*) and increasing concentrations of histone H2B

[H2B] (μM)	*R*			
	I1	I2	I3	Mean±SD
0.12	1.276×10^{-2}	1.255×10^{-2}	1.267×10^{-2}	$1.266\times10^{-2}\pm1.060\times10^{-4}$
0.24	3.294×10^{-2}	3.091×10^{-2}	3.269×10^{-2}	$3.218\times10^{-2}\pm1.107\times10^{-3}$
0.4	8.751×10^{-2}	8.712×10^{-2}	8.724×10^{-2}	$8.729\times10^{-2}\pm2.020\times10^{-4}$
1.2	3.079×10^{-1}	3.040×10^{-1}	3.100×10^{-1}	$3.073\times10^{-1}\pm3.045\times10^{-3}$
2.4	5.733×10^{-1}	5.621×10^{-1}	5.782×10^{-1}	$5.712\times10^{-1}\pm8.279\times10^{-3}$
4	7.100×10^{-1}	7.284×10^{-1}	6.986×10^{-1}	$7.123\times10^{-1}\pm1.505\times10^{-2}$
8	8.100×10^{-1}	8.205×10^{-1}	8.152×10^{-1}	$8.153\times10^{-1}\pm5.265\times10^{-3}$
16	9.321×10^{-1}	9.345×10^{-1}	9.307×10^{-1}	$9.324\times10^{-1}\pm1.922\times10^{-3}$
32	9.400×10^{-1}	9.493×10^{-1}	9.400×10^{-1}	$9.431\times10^{-1}\pm5.358\times10^{-3}$

The mean±SD (standard deviation) is calculated from the values obtained from three independent injections (I1, I2 and I3). *R*, saturation of the oligo. Analytical conditions as describes in Fig. 1A.

Table 1. In all the cases, variation among replicates is lower than 1%. Furthermore, given that the quantum yield of the method is constant for both the complex and uncomplexed oligo and the saturation of the oligo is a relative measurement, no-internal standard is required.

The equation describing single site binding is $R=L/(K_d+L)$ where K_d is the equilibrium dissociation constant for the complex, and L is the free concentration of the protein [18]. Assuming that the concentration of the protein is in excess over DNA throughout the experiment, as it is in the present case, then $R_{1/2}$ is an estimate of K_d. As anticipated, data fitting for both H4 and H2B to single-site binding curves using GraFit 3.1 software (Fig. 1B) was excellent (reduced χ^2 values of 0.001 and 0.002, respectively). $R_{1/2}$ for H4 was calculated to be 1.09 ± 0.3 μM and 2.43 ± 0.4 μM for H2B. Therefore, the affinity for DNA of H4 is higher than that of H2B, compatible with the lower isoelectric point of H2B. Both results are similar to other previously published data (between 1 and 10 μM) [19,20], thus corroborating the value of the method. In fact, it has been demonstrated the physiological relevance of this difference in affinity between these two histones. H3–H4 tetramers remain attached to DNA after the release of H2A–H2B dimers to facilitate transcription [21].

The method has also been demonstrated to be adequate for other DNA-binding proteins, such as the H2A and H3 histones, several methyl–DNA binding proteins and transcriptional factors [22], thus attesting to the wide-ranging nature of the procedure.

In summary, we have developed a new capillary electrophoretic mobility shift assay for protein–DNA binding that is reproducible and rapid and that can be used with small quantities of samples. The method boasts two main advances from previous HPCE-based methods for binding assays: deactivation of silanol groups so that protein adsorption onto the capillary walls is avoided, and the absence of any requirement for a gel matrix. These advantages generally provide trouble-free analyses and always avoid the putative undesirable effects of the sieving gel on the binding affinity. Furthermore, in a standard molecular biology laboratory, the advantage of HPCE over other accurate techniques for quantifying saturation fractions, such as microcalorimetry, is that it may be also employed in the study of other interesting molecular subjects, such as global DNA methylation [23–25], that are of special interest in human disease and specially in cancer research.

4. Notation

CEMSA	capillary electrophoretic mobility shift assay
EMSA	electrophoretic mobility shift assay
R	saturation fraction
RFU	relative fluorescence units
SSO	standard synthetic oligo

Acknowledgements

This work was supported by I+D+I project SAF 2001-0059 and the International Rett Syndrome Association. MF and EB are funded by a University educational staff Postdoctoral Fellowship and the Ramón y Cajal Program, respectively.

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