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Non-equilibrium capillary electrophoresis of equilibrium mixtures—appreciation of kinetics in capillary electrophoresis†

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We describe a new electrophoretic method (patent pending), Non-Equilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM), and demonstrate its application to the study of protein–DNA interactions. A single NECEEM experiment allows for the determination of equilibrium and kinetic parameters of protein–DNA complex formation. The equilibrium mixture is prepared by mixing protein and DNA; it contains three components: free protein, free DNA, and the protein–DNA complex. A small plug of such a mixture is injected onto a capillary and the three components are separated under non-equilibrium conditions using a run buffer that does not contain the components of the equilibrium mixture. The protein–DNA complex decays during the NECEEM separation; the resulting electropherograms contain characteristic peaks and exponential curves. A simple analysis of a single electropherogram reveals two parameters: the equilibrium dissociation constant of the protein–DNA complex and the monomolecular rate constant of complex decay. The bimolecular rate constant of complex formation can then be calculated as the ratio of the two experimentally-determined constants. NECEEM was applied to find the equilibrium and kinetic parameters of interaction between an *E. coli* single-stranded DNA binding protein and a fluorescently-labeled oligonucleotide. The constants determined by NECEEM are in good agreement with those obtained by other methods. The new method is simple, fast, and accurate. It can be equally applied to other non-covalent molecular complexes.

Introduction

Non-covalent protein–DNA complexes participate in gene expression, DNA replication, DNA integrity control, and DNA damage repair. In order to understand the dynamics of these biological processes it is important to know the *kinetic* parameters of the formation and decay of relevant protein–DNA complexes. The knowledge of these parameters is also essential for the development and optimization of analytical methods and molecular biology techniques based on protein–DNA interactions. The formation and decay of a protein–DNA complex, P·DNA, are characterized by a bimolecular rate constant, k_1, and a monomolecular rate constant, k_{-1}, respectively:

$$\mathrm{P + DNA} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{P \bullet DNA} \qquad (1)$$

The stability of the complex is often described in terms of the equilibrium dissociation constant:

$$K_\mathrm{d} = k_{-1}/k_1 \qquad (2)$$

Since k_1, k_{-1}, and K_d are related through the last expression, obtaining any pair of the three constants is sufficient to calculate a third one. For a protein–DNA complex, it is more practical to determine K_d and k_{-1} in experiment and then calculate k_1 from eqn. (2). Classically, K_d of a protein–DNA complex is obtained by electrophoresis mobility shift assay (EMSA) on slab gels.[1] EMSA has also been used to estimate k_{-1},[2] although the accuracy of this method is low and the dynamic range is very limited. More recently, surface plasmon resonance (SPR) has been successfully applied to measurements of K_d and k_{-1} for protein–DNA complexes.[3,4] Both EMSA on slab gels and SPR experiments require relatively large amounts of a protein.

When the quantity of a protein is a limiting factor, capillary electrophoresis (CE) has proved to be a technique of choice for studies of protein–DNA interactions. A CE analogue of slab-gel EMSA (CEMSA) has been developed and applied to the determination of K_d for protein–DNA complexes.[5–8] For rapidly decaying protein–DNA complexes, affinity capillary electrophoresis (ACE) of the complex can be used to prevent complex decay during the separation.[9,10] In ACE, the run buffer is supplemented with one of the components of the complex to maintain the equilibrium during separation. The assumption of equilibrium in CEMSA and ACE allows for the determination of K_d but makes finding k_{-1} difficult. Here we propose non-equilibrium capillary electrophoresis of the equilibrium mixtures (NECEEM) of a protein, DNA, and a protein–DNA complex as a fast method to find both K_d and k_{-1} in a single experiment. We used the new method to study the interaction between an *E. coli* single-stranded DNA binding protein (SSB) and a fluorescently-labeled 15-mer oligonucleotide (fDNA). A short communication on the method described here was published in the *Journal of the American Chemical Society*.[11]

Experimental

Reagents and materials

E. coli single-stranded DNA binding protein (SSB), and buffer components were from Sigma-Aldrich (Oakville, ON). A fluorescently-labeled 15-mer oligonucleotide, 5′-fluorescein-GCGGAGCGTGGCAGG (fDNA), was kindly donated by Dr. Yingfu Li (McMaster University, Hamilton, ON). Fused-silica capillaries were purchased from Polymicro (Phoenix, AZ). Deionized water of Milli-Q quality was used for all solutions.

† Electronic supplementary information (ESI) available: Determination of K_d for non-covalent interaction between a protein and DNA, and determination of the reletive quantum yield of fDNA in free and SSB-bound forms. See http://www.rsc.org/suppdata/an/b2/b212913b/

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Capillary electrophoresis

NECEEM separation of protein–DNA complexes was performed using a laboratory-built CE instrument with a post-column fluorescence detector described in detail elsewhere.[12] Uncoated fused-silica capillaries of 40 cm × 20 μm id × 150 μm od were used in all experiments. Electrophoresis was run in a positive-polarity mode (positive electrode at the injection end) using a Spellman CZE 1000 power supply (Plainview, NY, USA) as a source of high voltage. A 488 nm line of an Ar-ion laser (Melles Griot, Ottawa, ON) was utilized to excite fluorescence of fDNA. Fluorescence was filtered from stray and scattered laser light with a band pass filter centered at 520 nm (Omega Optical, Brattleboro, VT). An R1477 photomultiplier tube (Hamamatsu, Middlesex, NJ) was used as a fluorescence light detector.

Fluorescence anisotropy was measured with the same CE instrument slightly modified as described elsewhere.[10] The value of anisotropy was calculated according to the following expression:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (3)$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of fluorescence in the planes parallel and perpendicular to the plane of polarization of excitation light.

Three run buffers were used for NECEEM: 25.0 mM, 16.7 mM and 12.5 mM borate at pH 9.4. The run buffer for ACE was 25.0 mM borate at pH 9.4 supplemented with 1 μM SSB. The samples were injected into the capillary by a pressure pulse of 1 s × 9.1 kPa; the length of corresponding sample plug was 0.93 mm as was calculated using the Poiseulle equation. The electrophoresis was carried out with an electric field of 600 V cm^{-1} at ambient temperature. The capillary was rinsed with the run buffer solution for 2 min prior to each run. At the end of each run, the capillary was rinsed with 100 mM NaOH for 2 min, followed by a rinse with deionized water for 2 min.

Equilibrium mixtures

To prepare an equilibrium mixture of the protein, DNA and the protein–DNA complex, we mixed solutions of 16 μM SSB and 205 nM fDNA in the NECEEM run buffer at a desired volume ratio and incubated at room temperature to reach the equilibrium prior to the analysis.

Results and discussion

NECEEM

Equilibrium mixtures contained three components: free SSB, free fDNA and the SSB·fDNA complex. NECEEM of such mixtures generated electropherograms with three essential features: peaks 1 and 2 and curved line 3 (Fig. 1). Sole peak 1 was observed when SSB was not present in the equilibrium mixture, indicating that peak 1 represents fDNA (Fig. 1A). When the concentration of SSB was intermediate, all three features were observed (Fig. 1B). The height of peak 1 decreased with increasing concentration of SSB. When the concentration of SSB was saturating, peak 1 was not observed (Fig. 1C). Curve 3 was shown to be perfectly fitted by a single exponential line and therefore will be called an "exponential part" of the electropherogram. To reveal the identities of the three features in the electropherograms we need to consider the fate of the three components (free SSB, free fDNA, and the SSB·fDNA complex) while being separated by NECEEM. Both SSB and fDNA were negatively charged under our conditions resulting in negative electrophoretic mobilities for both free SSB and free fDNA. However, the negative charge to size ratio was lower for SSB; therefore, the electrophoretic mobility of free SSB was higher than that of free fDNA. The SSB·fDNA complex had an intermediate value of electrophoretic mobility. Because of these differences in the electrophoretic mobilities, the equilibrium fractions of free fDNA and free SSB were removed from the electrophoretic zone of the SSB·fDNA complex as soon as electrophoresis started. The equilibrium fraction of free fDNA migrated as a single electrophoretic zone and resulted in peak 1 with the longest migration time, as was confirmed by sampling pure fDNA. The equilibrium fraction of free SSB also migrated as a single zone, but due to the lack of a fluorescent label, free SSB did not contribute to the electropherograms. The equilibrium fraction of the SSB·fDNA complex could not generate a single electrophoretic peak since the equilibrium of the complex was not maintained in NECEEM. The complex continuously decayed during the separation resulting in the non-equilibrium production of free fDNA and free SSB. According to eqn. (1), the rate of fDNA production reduced exponentially following the monomolecular decay of the complex during NECEEM separation:

$$\frac{\mathrm{d[fDNA]}}{\mathrm{d}t} = -\frac{\mathrm{d[SSB \bullet fDNA]}}{\mathrm{d}t} = [\mathrm{SSB \bullet fDNA}]_{eq} \exp(-k_{-1}t) \quad (4)$$

Here $[SSB \cdot fDNA]_{eq}$ is the equilibrium concentration of the complex in the equilibrium mixture, k_{-1} is the monomolecular

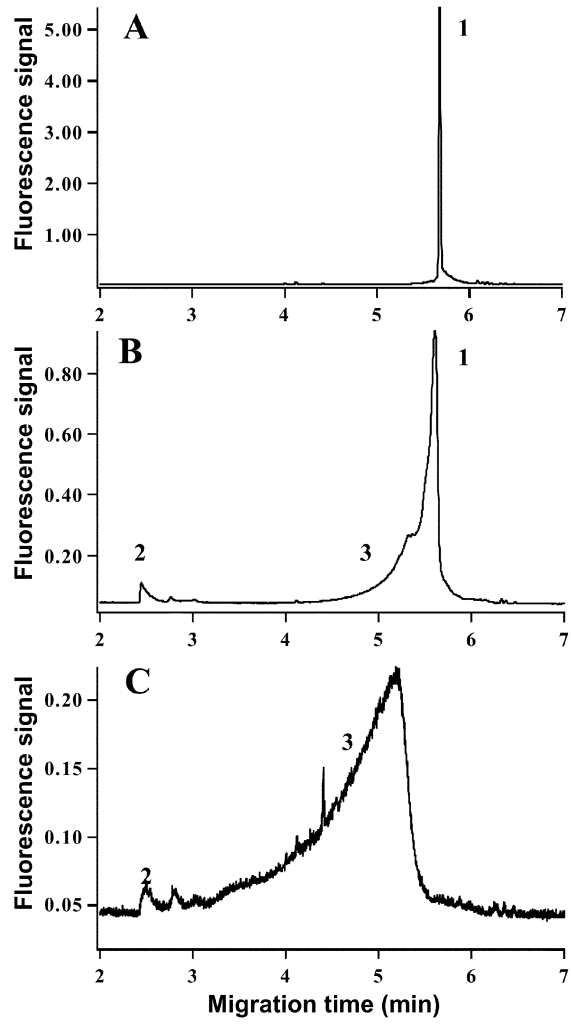

Fig. 1 NECEEM of SSB and fDNA. The total concentrations of SSB and fDNA in the mixtures were: (A) $[SSB]_0$ = 0, $[fDNA]_0$ = 0.20 μM; (B) $[SSB]_0$ = 0.32 μM, $[fDNA]_0$ = 0.16 μM; (C) $[SSB]_0$ = 0.80 μM, $[fDNA]_0$ = 0.10 μM. The run buffer was 25.0 mM borate at pH 9.4.

rate of complex decay and t is the time passed from the beginning of the separation. Exponential part 3 of the electropherograms reflects the production of free fDNA, or in other words, the decay of the SSB·fDNA complex. Peak 2 corresponds to the SSB·fDNA complex that remained intact at the time of its elution from the capillary. This was confirmed by increasing peak 2 with decreasing separation time (Fig. 2A). During a shorter separation time a lesser fraction of the complex decayed and a larger fraction of the complex exited the capillary intact.

The identities of the features in the electropherograms were also confirmed by measuring the fluorescence anisotropy of fDNA in the two peaks and the exponential part (Fig. 2B). The fDNA molecule is much smaller than the SSB·fDNA complex; therefore the anisotropy of free fDNA is lower than that of the complex. Peak 1 and exponential part 3 had identical small anisotropy, $r_{fDNA} = 0.03$, corresponding to free fDNA, while peak 2 had a higher anisotropy, $r_{SSB\cdot fDNA} = 0.11$, corresponding to the SSB·fDNA complex. It should be emphasized that NECEEM provides a unique way of directly measuring the fluorescence anisotropy of the complex; the electrophoretic zone corresponding to peak 2 contains no free fDNA. We used the values of r_{fDNA} and $r_{SSB\cdot fDNA}$ in our determination of the relative quantum yield of SSB-bound fDNA (see below).

Determination of K_d

Our assignment of peak 1 to the equilibrium fraction of free fDNA and both peak 2 and exponential part 3 to the equilibrium fraction of the SSB·fDNA complex allowed us to calculate the equilibrium dissociation constant K_d. The equilibrium fraction of free fDNA is proportional to the area of peak 1, A_1:

$$[\text{fDNA}]_{eq} = A_1\, a/\varphi_{fDNA} \quad (5)$$

where a is a constant, and φ_{fDNA} is the quantum yield of fluorescence of free fDNA. The equilibrium fraction of the complex is dependent on the areas of peak 2, A_2, and exponential part 3, A_3:

$$[\text{SSB·fDNA}]_{eq} = A_2\, a/\varphi_{SSB\cdot fDNA} + A_3\, a/\varphi_{fDNA} \quad (6)$$

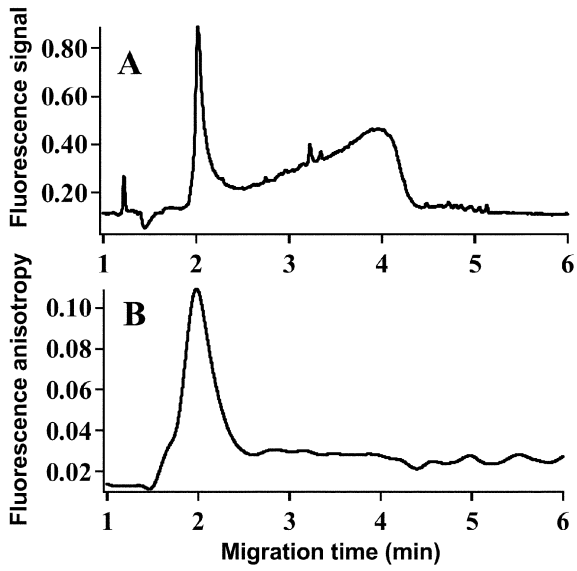

Fig. 2 NECEEM of SSB and fDNA with shortened separation time (compare to Fig. 1). Shortening of the separation time was achieved by increasing the velocity of electroosmotic flow; the velocity was increased through decreasing the ionic strength of the run buffer. Panel A shows the total fluorescence intensity while panel B demonstrates the corresponding fluorescence anisotropy. The total concentrations of the protein and fDNA in the mixture were: $[\text{SSB}]_0 = 1.30$ μM and $[\text{fDNA}]_0 = 0.13$ μM. The run buffer was 12.5 mM borate at pH 9.4.

where $\varphi_{SSB\cdot fDNA}$ is the quantum yield of fluorescence of the complex. Using eqns. (5) and (6) we can find the ratio, R, of the two equilibrium fractions:

$$R = \frac{[\text{fDNA}]_{eq}}{[\text{SSB}\bullet\text{fDNA}]_{eq}} = \frac{A_1}{A_2\varphi_{fDNA}/\varphi_{SSB\bullet fDNA} + A_3} \quad (7)$$

On the other hand the knowledge of this ratio is sufficient for the determination of K_d (see ESI†):

$$K_d = \frac{[\text{SSB}]_0(1+R) - [\text{fDNA}]_0}{1 + 1/R} \quad (8)$$

To determine R we need to find the relative quantum yield, $\varphi_{fDNA}/\varphi_{SSB\cdot fDNA}$, and the three areas, A_1, A_2, and A_3. The relative quantum yield was found to be 0.95 ± 0.03 (see the next section). The areas A_1, A_2, and A_3 were calculated as demonstrated in Fig. 3. Eqns. (7) and (8) were then used to calculate the value of K_d equal to 280 ± 50 nM based on six experiments with different concentrations of SSB and fDNA mixed. This value is in agreement with those obtained by other methods: 230 nM and 200 nM for binding of SSB with an 11-mer oligonucleotide measured by ACE and fluorescence anisotropy, respectively,[9] and 710 nM for binding of SSB with a 16-mer oligonucleotide measured by the fluorescence quenching method.[13] The differences between the K_d values measured by different methods can be ascribed to differences in oligonucleotide length and to the use of different buffers.

Determination of relative quantum yield

The value of the relative quantum yield of fluorescence of fDNA, $\varphi_{fDNA}/\varphi_{SSB\cdot fDNA}$, was found by comparing peak areas generated by free fDNA and SSB-bound fDNA. Fluorescence of free fDNA was measured in a CE experiment with no SSB in the sample or in the run buffer (Fig. 4A). Fluorescence of SSB-bound fDNA was measured in an ACE experiment with the run buffer containing SSB (Fig. 4B). Fluorescein, which does not interact with SSB, was used as an internal marker in both experiments to normalize the fluorescence intensities of fDNA. The presence of SSB in the run buffer maintained the equilibrium between free fDNA and SSB-bound fDNA during the ACE separation. The relative quantum yield of SSB-bound fDNA was calculated using the following expression (see ESI†):

$$\frac{\varphi_{fDNA}}{\varphi_{SSB\bullet fDNA}} = \frac{1 - (r_{SSB\bullet fDNA} - r_{ACE})/(r_{SSB\bullet fDNA} - r_{fDNA})}{A_{ACE}/A_{CE} - (r_{SSB\bullet fDNA} - r_{ACE})/(r_{SSB\bullet fDNA} - r_{fDNA})} \quad (9)$$

where A_{ACE} and A_{CE} are peak areas generated by the same amount of fDNA in the ACE and CE experiments, respectively; r_{ACE} is the fluorescence anisotropy in the ACE experiment; the

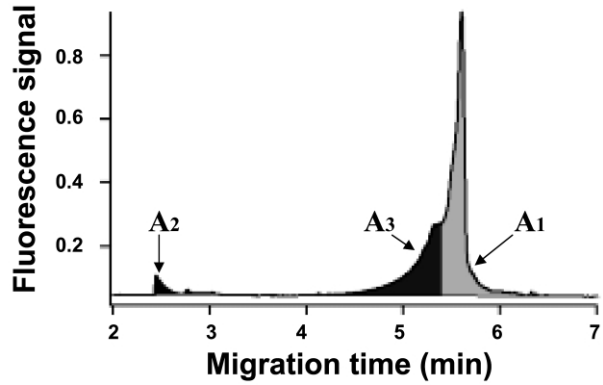

Fig. 3 Determination of areas A_1 and $A_2 + A_3$ required for finding the equilibrium dissociation constant K_d of the SSB·fDNA complex from a single NECEEM experiment.

values of fluorescence anisotropy of free fDNA, r_{fDNA}, and SSB-bound fDNA, $r_{SSB\cdot fDNA}$, were determined in NECEEM experiments (see above). The relative quantum yield of fluorescence, $\varphi_{fDNA}/\varphi_{SSB\cdot fDNA}$, was found to be equal to 0.95 ± 0.03 (n = 6). We expected that this value would be close to unity since the fluorescein moiety was not involved directly into SSB–fDNA interactions. In general the $\varphi_{fDNA}/\varphi_{SSB\cdot fDNA}$ ratio in eqn. (7) can be assumed to be equal to 1 unless fDNA is labeled with a fluorophore whose fluorescence can be quenched by a protein.[13]

Whether or not the quantum yield of fDNA changes upon binding to SSB can be examined in a fast fashion in NECEEM experiments with varying separation time. If the separation time shortens then more of the intact SSB·fDNA complex reaches the detection end of the capillary. Thus, area A_2, which corresponds to the complex, increases, while area A_3 decreases. If the total area $A_1 + A_2 + A_3$ does not change then the quantum yield of fDNA fluorescence is not affected upon binding to SSB. We decreased the separation time by using run buffers with decreasing ionic strengths. Fig. 5 shows the observed changes in the electrophoretic features. The total area, $A_1 + A_2 + A_3$, did not change confirming that the quantum yield of fDNA was not affected by binding to SSB.

Determination of k_{-1} and k_1

The monomolecular rate constant of complex decay, k_{-1}, can be determined from exponential part 3 of the electropherogram by fitting the experimental data (Fig. 6) with a single exponential function:

$$I_t = I_{t_0} \exp\left\{k_{-1}\frac{t_{SSB\bullet fDNA}}{t_{fDNA} - t_{SSB\bullet fDNA}}(t - t_0)\right\} \quad (10)$$

where I_t and I_{t_0} are the fluorescence intensities at times t and t_0, respectively, and t_{fDNA} and $t_{SSB\cdot fDNA}$ are migration times of fDNA and SSB·fDNA, respectively. The $t_{SSB\cdot DNA}/(t_{fDNA} - t_{SSB\cdot DNA})$ coefficient reflects the apparent shortening of the time window in which the complex decay is monitored: $t_{SSB\cdot fDNA}$ to t_{fDNA} instead of 0 to t_{fDNA}. The value of k_{-1} for the decay of the SSB·fDNA complex was determined to be (3.3 ± 1.6) × 10^{-2} s^{-1} (n = 6). Using eqn. (2) and the found values of K_d and k_{-1} we calculated the bimolecular rate constant of the SSB·fDNA complex formation, k_1 = (11.7 ± 5.6) × 10^4 $M^{-1}s^{-1}$ (n = 6). It is worthwhile to emphasize that the values of K_d and k_{-1} were determined from a single electropherogram.

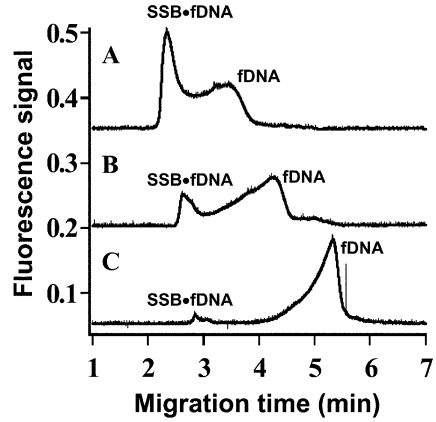

Fig. 5 The influence of the separation time on the electropherograms generated by NECEEM. The separation time was changed by changing the electroosmotic velocity through using run buffers with different ionic strengths: 12.5 mM Borax pH 9.4 (A), 16.7 mM Borax pH 9.4 (B) and 25.0 mM Borax pH 9.4 (C). Although the relative intensities of the electrophoretic features changed, the total area of the fluorescence signal remained constant with changing separation time. The total concentrations of the protein and fDNA in the equilibrium mixture were $[SSB]_0$ = 0.80 μM and $[fDNA]_0$ = 0.10 μM. The electropherograms are offset for the clarity of presentation.

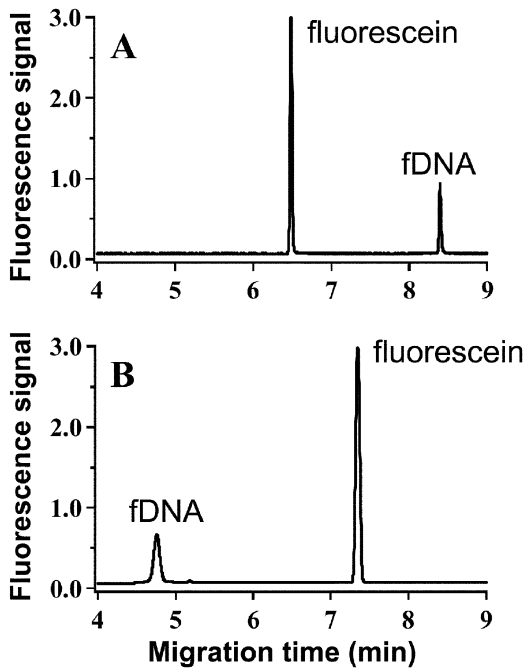

Fig. 4 Analysis of the same amount of fDNA (0.10 μM) by CE (panel A) and by ACE (panel B). In both experiments, the solution of fDNA contained 0.25 μM fluorescein used as an internal marker. In CE, the run buffer was 25.0 mM borate at pH 9.4; in ACE, the run buffer was 25.0 mM borate supplemented with 1.0 μM SSB. The areas of the peaks were identical in the two experiments confirming that the quantum yield of fDNA did not change upon binding to SSB.

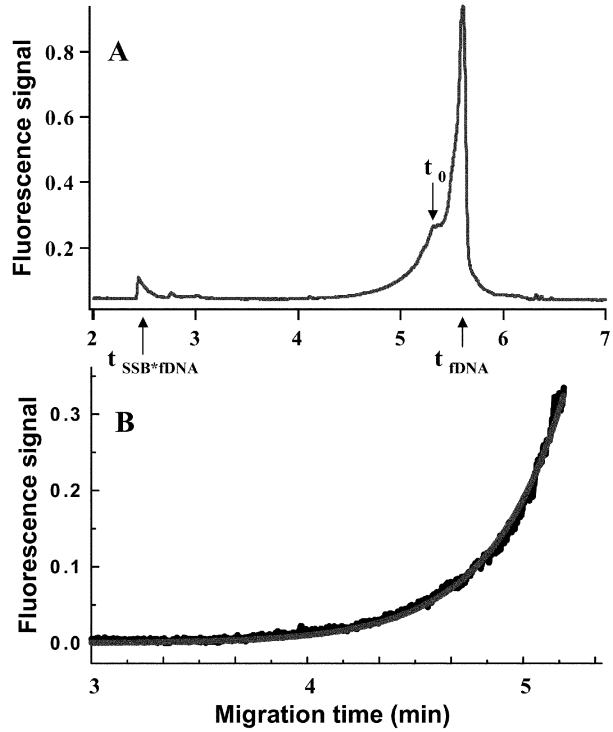

Fig. 6 The value of k_{-1} can be found by fitting the exponential part of the electropherogam with a single exponential function, represented by eqn. (10). Frame A shows the whole electropherogram and illustrates the assignment of times t_0, t_{fDNA} and $t_{SSB\cdot fDNA}$. Frame B shows the best fit of experimental data with a single exponential function.

If the monomolecular rate constant of complex decay, k_{-1}, is too small or separation of the two peaks is not sufficient to resolve the exponential part, then the value of k_{-1} can be determined by measuring the area of peak 2 (corresponding to the amount of the protein–DNA complex) as a function of peak 2 migration time. Migration time can be varied by changing one or more of the following 4 parameters: (i) capillary length, (ii) electric field, (iii) run buffer composition, and (iv) differential pressure applied to capillary ends. The area will decrease with increasing time exponentially, and the value of k_{-1} can be calculated from this data.

Conclusions

NECEEM takes the advantage of a rapidly decaying complex to calculate equilibrium and kinetic parameters of complex formation and decay in a single experiment. Knowledge of these constants is essential for understanding the dynamics of regulatory biological processes. Only slight modification of the NECEEM separation conditions may be needed to optimize this method for another protein–DNA complex. The parameters to be optimized are: the buffer concentration, the capillary length, the strength of the electric field used for NECEEM. Another unique feature of NECEEM is its extremely high sensitivity. We were able to determine the parameters of protein–DNA interaction with the amount of protein as low as 10^{-18} moles. The next exciting applications of NECEEM will include extremely sensitive analyses of proteins using oligonucleotide aptamers as affinity probes.

Acknowledgement

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Supplementary Material

Determination of K_d for non-covalent interaction between a protein and DNA

Here we derive the expression for K_d, as a function of the total concentrations of protein and DNA and the ratio between the equilibrium concentrations of free protein and protein-DNA complex.

Lets consider the following equilibrium between SSB, fDNA, and their complex, SSB•fDNA:

$$\mathrm{SSB + fDNA} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{SSB{\bullet}fDNA} \qquad (1s)$$

Lets assume that the ratio, R, of equilibrium concentrations of free fDNA, $[\mathrm{fDNA}]_{eq}$, and the complex, $[\mathrm{SSB{\bullet}fDNA}]_{eq}$, is known from measuring the areas of peaks on the electropherogram:

$$\mathrm{R} = \frac{[\mathrm{fDNA}]_{eq}}{[\mathrm{SSB} \bullet \mathrm{fDNA}]_{eq}} \qquad (2s)$$

From expression (2s) we get:

$$[\mathrm{SSB} \bullet \mathrm{fDNA}]_{eq} = \frac{[\mathrm{fDNA}]_{eq}}{\mathrm{R}} \qquad (3s)$$

Using the conservation of mass principle for fDNA and expression (3s) we obtain:

$$[\mathrm{fDNA}]_0 = [\mathrm{fDNA}]_{eq} + [\mathrm{SSB} \bullet \mathrm{fDNA}]_{eq} = [\mathrm{fDNA}]_{eq} + [\mathrm{fDNA}]_{eq}/\mathrm{R} = [\mathrm{fDNA}]_{eq}(1 + 1/\mathrm{R}) \qquad (4s)$$

From expression (4s) we can derive:

$$[\mathrm{fDNA}]_{eq} = \frac{[\mathrm{fDNA}]_0}{(1 + 1/\mathrm{R})} = \frac{[\mathrm{fDNA}]_0 \mathrm{R}}{(1 + \mathrm{R})} \qquad (5s)$$

From the conservation of mass principle for fDNA and expression (5s) we get:

$$[\mathrm{SSB} \bullet \mathrm{fDNA}]_{eq} = [\mathrm{fDNA}]_0 - [\mathrm{fDNA}]_{eq} = [\mathrm{fDNA}]_0 - \frac{[\mathrm{fDNA}]_0 \mathrm{R}}{(1 + \mathrm{R})} = [\mathrm{fDNA}]_0 \left(1 - \frac{\mathrm{R}}{1 + \mathrm{R}}\right) =$$

$$= [\mathrm{FDNA}]_0 \left(\frac{1}{1 + \mathrm{R}}\right) = \frac{[\mathrm{FDNA}]_0}{1 + \mathrm{R}} \qquad (6s)$$

Using the conservation of mass principle for SSB and expression (6s) we can write:

$$[\mathrm{SSB}]_{eq} = [\mathrm{SSB}]_0 - [\mathrm{SSB} \bullet \mathrm{fDNA}]_{eq} = [\mathrm{SSB}]_0 - \frac{[\mathrm{fDNA}]_0}{1 + \mathrm{R}} = \frac{[\mathrm{SSB}]_0 (1 + \mathrm{R}) - [\mathrm{fDNA}]_0}{1 + \mathrm{R}} \qquad (7s)$$

Finally, from the definition of K_d and expressions (5s)-(7s) we derive:

$$\boldsymbol{K}_d = \frac{[\mathrm{SSB}]_{eq}[\mathrm{fDNA}]_{eq}}{[\mathrm{SSB} \bullet \mathrm{fDNA}]_{eq}} = \frac{\frac{[\mathrm{SSB}]_0 (1 + \mathrm{R}) - [\mathrm{fDNA}]_0}{1 + \mathrm{R}} \times \frac{[\mathrm{fDNA}]_0 \mathrm{R}}{1 + \mathrm{R}}}{\frac{[\mathrm{fDNA}]_0}{(1 + \mathrm{R})}} = \frac{[\mathrm{SSB}]_0 (1 + \mathrm{R}) - [\mathrm{fDNA}]_0}{1 + 1/\mathrm{R}} \qquad (8s)$$

Determination of the relative quantum yield of fDNA in free and SSB-bound forms.

fDNA is present only in the free form in CE; therefore, the peak area in the CE experiment is:

$$A_{CE} = b\,\varphi_{fDNA} \tag{9s}$$

where b is a constant.

The presence of SSB in the run buffer during ACE results in the equilibrium between free and SSB-bound forms of fDNA. Since the quantum yield of fluorescence is an additive function, the peak area of fDNA in ACE is the sum of two components corresponding to free fDNA and SSB-bound fDNA:

$$A_{ACE} = b\left\{\varphi_{fDNA}\frac{[fDNA]_{eq}}{[fDNA]_0} + \varphi_{SSB\bullet fDNA}\frac{[SSB\bullet fDNA]_{eq}}{[fDNA]_0}\right\} \tag{10s}$$

Using the conservation of mass principle for fDNA and expression (10s) we get:

$$A_{ACE} = b\left\{\varphi_{fDNA}\frac{[fDNA]_{eq}}{[fDNA]_0} + \varphi_{SSB\bullet fDNA}\left(1-\frac{[fDNA]_{eq}}{[fDNA]_0}\right)\right\} \tag{11s}$$

Dividing expression (11s) by (9s) and rearranging the result leads to the desired expression:

$$\frac{\varphi_{fDNA}}{\varphi_{SSB\bullet fDNA}} = \frac{1-[fDNA]_{eq}/[fDNA]_0}{A_{ACE}/A_{CE}-[fDNA]_{eq}/[fDNA]_0} \tag{12s}$$

The fraction on non-bound fDNA, $[fDNA]_{eq}/[fDNA]_0$, can be found from fluorescence anisotropy experiments. Similarly to the quantum yield, fluorescence anisotropy is an additive function. Thus, the anisotropy in the ACE experiment is a sum of two components corresponding to free and SSB-bound fDNA:

$$r_{ACE} = r_{fDNA}\frac{[fDNA]_{eq}}{[fDNA]_0} + r_{SSB\bullet fDNA}\frac{[SSB\bullet fDNA]_{eq}}{[fDNA]_0} \tag{13s}$$

Using the conservation of mass principle for fDNA and expression (13s) we get:

$$r_{ACE} = r_{fDNA}\frac{[fDNA]_{eq}}{[fDNA]_0} + r_{SSB\bullet fDNA}\left(1-\frac{[fDNA]_{eq}}{[fDNA]_0}\right) \tag{14s}$$

Rearranging of expression (14s) leads gives a formula for the fraction of non-bound fDNA:

$$\frac{[fDNA]_{eq}}{[fDNA]_0} = \frac{r_{SSB\bullet fDNA}-r_{ACE}}{r_{SSB\bullet fDNA}-r_{fDNA}} \tag{15s}$$

Finally, by substituting (15s) into (12s) we get an expression for the relative quantum yield as a function of peak areas and fluorescence anisotropy:

$$\frac{\varphi_{fDNA}}{\varphi_{SSB\bullet fDNA}} = \frac{1-(r_{SSB\bullet fDNA}-r_{ACE})/(r_{SSB\bullet fDNA}-r_{fDNA})}{A_{ACE}/A_{CE}-(r_{SSB\bullet fDNA}-r_{ACE})/(r_{SSB\bullet fDNA}-r_{fDNA})}$$