Slow-Dissociation and Slow-Recombination Assumptions in Nonequilibrium Capillary Electrophoresis of Equilibrium Mixtures

Svetlana M. Krylova, Peter M. Dove, Mirzo Kanoatov, and Sergey N. Krylov*

Department of Chemistry and Centre for Research on Biomolecular Interactions, York University, Toronto, Ontario M3J 1P3, Canada

**ABSTRACT:** Nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) is a kinetic affinity method with both analytical and preparative applications. NECEEM requires that the dissociation of the complexes be negligible in its first phase and the recombination of the dissociated complexes be negligible in its second phase. Here, we introduce a method, which facilitates easy examination of whether or not these requirements are satisfied. We derived expressions for two parameters, termed the slow-dissociation parameter (SDP) and slow-recombination parameter (SRP), which can be used to assess the assumptions. Both parameters should be much less than 1 for the assumptions to be satisfied. We calculated the two parameters for new and previously published NECEEM experiments and found that the assumptions were satisfied in all of them. Finally, we discuss changes to NECEEM conditions that should be done if the assumptions are found not to be satisfied. The SDP/SRP assessment helps to easily validate the results of NECEEM-based analyses and thus makes the NECEEM method more robust.

Nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) is the most popular and arguably the most versatile method of kinetic capillary electrophoresis (KCE). NECEEM is used for measurements of equilibrium and rate constants of affinity interactions. It was applied to a variety of interacting pairs of different nature including protein—DNA, protein—peptide, protein—DNA-encoded small molecule, protein—small molecule, and polyvinyl alcohol—fluorescent dye. NECEEM was also used to measure unknown concentration of molecules in a sample with a labeled affinity probe. Finally, NECEEM became an indispensable tool in the high-efficiency selection of DNA aptamers. In this application, NECEEM guarantees an unbeatable efficiency in the partitioning of protein-bound DNA from unbound DNA. Not only does NECEEM allow faster selection of aptamers but also it can facilitate selection of smart aptamers (aptamers with desirable binding parameters).

NECEEM typically deals with noncovalent interactions of two molecules, A and B, that together form a complex C:

\[
\begin{align*}
A + B & \rightarrow_{k_+} C \\
K_d & = k_- / k_+ 
\end{align*}
\]

where \(k_+\) and \(k_-\) are rate constants of complex formation and dissociation, respectively, and \(K_d\) is the equilibrium dissociation constant. It facilitates direct measurements of \(k_-\) and \(K_d\) while \(k_+\) is calculated as \(k_- / K_d\). An equilibrium mixture containing the three components, A, B, and C, is prepared, and a short plug of this equilibrium mixture is injected into a capillary (Figure 1). Electrophoresis is then used for the continuous separation of the A, B, and C components. In the first phase, the equilibrium fractions of A, B, and C are separated. The amounts of each component present in the corresponding equilibrium fraction can be used to calculate \(K_d\). When A and B are removed from the zone of C, C is no longer at equilibrium with A and B and starts to dissociate. The zone of C becomes the dissociation zone. In the second longer phase, A and B which are formed from the dissociating C are continuously removed from the dissociation zone. Dissociation of C follows monomolecular kinetics, and the amount of A (or B) released over a known period of time can be used to find \(k_-\).

NECEEM uses two assumptions. The first assumption is that the dissociation of C is negligible in the first phase; this assumption is required for accurate determination of \(K_d\). The second assumption is that the recombination of A and B is negligible in the second phase; this assumption is required for accurate determination of \(k_-\).

Currently, there is no way to easily understand whether or not the two assumptions are satisfied. Qualitative analysis of NECEEM electropherograms cannot serve as a conclusive source of information. NECEEM electropherograms qualitatively depend on multiple parameters including concentrations of A and B, \(k_+\) and \(k_-\), velocities of A, B, and C, and lengths of the injected plug and the capillary. Changing any single parameter will change the NECEEM electropherogram in a unique way. Changing several parameters simultaneously will further complicate the electropherogram’s “response”. Therefore, it is very difficult to find a specific qualitative feature in the electropherograms that would indicate whether or not the two assumptions are satisfied.
This problem motivated us to derive simple quantitative parameters that depend on the concentrations, rate constants, velocities, and lengths and could suggest whether or not the two major assumptions of NECEEM are satisfied.

Here, we introduce two such parameters, the slow-dissociation parameter (SDP) and the slow-recombination parameter (SRP). If these parameters are much smaller than unity, then the two major assumptions of NECEEM are satisfied. Finally, we illustrate the use of the SDP and the SRP experimentally and apply them to examine new and previously published NECEEM results.\textsuperscript{2,11,12}

\section*{EXPERIMENTAL SECTION}

All NECEEM procedures were performed using the following instrumental setup. Capillary electrophoresis (CE) was carried out with a P/ACE MDQ apparatus (Beckman Coulter, Mississauga, ON, Canada) equipped with a fluorescence detector; a 488 nm line of continuous Wave Solid-State laser (JDSU, Santa Rosa, CA) was utilized to excite the fluorescence. Uncoated fused-silica capillaries with an inner diameter of 75 \( \mu \text{m} \) and outer diameter of 360 \( \mu \text{m} \) were used. Runs were performed in a 50 cm-long (40 cm to the detection window) capillary. Both the inlet and the outlet reservoirs contained the electrophoresis run buffer (25 mM Borax at pH 9.2). At the end of each run, the capillary was sequentially rinsed with 100 mM HCl, 100 mM NaOH, and deionized water for 2 min with an applied pressure of 20 psi. The samples were injected into the capillary, prefilled with the run buffer, with a pressure pulse of 0.5 psi for 6 s. The length of the sample plug was calculated to be 6.8 mm. Electrophoresis was carried out with a positive electrode at the injection end of the sample plug was calculated to be 6.8 mm. Electrophoresis was carried out with a positive electrode at the injection end of the capillary; the direction of the electroosmotic flow was from the inlet to the outlet reservoir. Separation was carried out by an electric field of 500 V cm\(^{-1}\). The temperature of the capillary was maintained at 15 \( ^\circ \text{C} \) during the separation.

To obtain the experimental data, the interaction between the AlkB protein from \textit{E. coli} and a DNA aptamer was studied. Equilibrium mixtures were prepared with electrophoresis run buffer and contained 120 nM AlkB protein, 30 nM DNA aptamer, and 20 nM Bodipy (internal standard). The DNA aptamer was fluorescently labeled for detection. Integrated signal area of the internal standard was used for normalization of signal intensities and migration times between runs. Associated peak areas and migration times were obtained from the resulting electropherograms and used to calculate equilibrium and rate constants with the following equations:

\[
K_d = \frac{B_0 - A_0}{A_0} \left( 1 - \frac{S_A}{S_A + S_{C, \text{int}} + S_{C, \text{dis}}} \right) \frac{S_A}{S_A + S_{C, \text{int}} + S_{C, \text{dis}}} - 1
\]

\[
k_- = \ln \left( \frac{S_A + S_{C, \text{dis}}}{S_A} \right) \frac{t_1}{t_C}
\]

\[
k_+ = \frac{k_-}{K_d}
\]

where \( B_0 \) and \( A_0 \) are the initial concentrations of AlkB protein and DNA aptamer after mixing but prior to complex formation, respectively; \( S_A, S_{C, \text{int}} \), and \( S_{C, \text{dis}} \) are integrated signal areas of free aptamer peak, AlkB–aptamer complex peak, and exponential decay region, respectively; and \( t_C \) is the migration time of the AlkB–aptamer complex peak to the detector.

\section*{RESULTS AND DISCUSSION}

\textbf{Phase 1: Separation of the Equilibrium Fractions.} The goal of the first phase in NECEEM is to remove at least one unbound fraction (either A or B) from the spatial zone of C before any significant amount of C dissociates. This removal would allow us to determine the equilibrium fractions of at least two components of the equilibrium mixture which is required for finding \( K_d \). This removal is also required to start phase 2. If \( l \) is the length of the injected plug containing the equilibrium mixture, we can assume that in phase 1 the length of the C zone is also \( l \). In addition, if we define the velocity of \( A, v_A \), to differ from that of \( C, v_C \), more than the velocity of \( B, v_B \), differs from \( v_C \): \( |v_A - v_C| > |v_B - v_C| \), then the removal of A from the C zone defines the duration of phase 1:

\[
t_1 = |v_A - v_C|^{-1}
\]

The maximum change in the concentration of \( C, \Delta C \), due to its dissociation during phase 1 can be related to the equilibrium concentration, \( C_{eq} \), through the following expression:

\[
\Delta C < C_{eq} k_{-1} t_1
\]

where \( C_{eq} k_{-1} \) is the initial (and thus maximal) rate of dissociation of C. This change should be much smaller than \( C_{eq} \) for the assumption of slow dissociation to be satisfied:

\[
\Delta C < C_{eq}
\]

Using inequalities (2) and (3), we can write a stronger condition for the slow-dissociation assumption:

\[
C_{eq} k_{-1} t_1 < C_{eq}
\]

Using eq 1, inequality (4) can be expressed as:

\[
SDP = k_+ |v_A - v_C|^{-1} < 1
\]

where we define the slow dissociation parameter (SDP) as \( k_+ |v_A - v_C|^{-1} \). Inequality (5) is a condition which must be satisfied in order for the slow-dissociation assumption to be valid.

SDP is a dimensionless parameter whose value is defined by how fast the initial separation of zones occurs. SDP can be changed by varying the length of the injected plug of equilibrium mixture and/or the difference between the velocities of A and C.

In order to determine the SDP, the linear speeds through the capillary must be known for both the complex C and the unbound component which separates from C the fastest (A in the above definition). Finding these velocities is usually not a problem. The molecule, which is separated from C fastest, is
typically labeled for detection (e.g., by a fluorophore), as a result both A and C are “visible”.

In cases where the SDP is close to 1, several changes may be made to the experimental procedure to lower the parameter and hence improve the validity of the results. The first and potentially easiest approach is to reduce the length of the injected plug, 𝑙. Obviously, this will result in less sample being introduced and may require a more concentrated sample mixture, which may be prohibitive. The second option is to adjust the CE conditions (e.g., buffer composition/concentration or the electric field strength) to maximize the difference in velocities.

**Phase 2: Dissociation of C After Removal of Equilibrium Fractions of A.** In phase 2, A is continuously removed from C to keep C out of equilibrium and force it to continuously dissociate. The main assumption of phase 2 is that there is negligible recombination of A and B which are produced by the dissociation of C. The recombination is impossible after the zone of A is separated from the zone of B. The time required for the phase 2 separation is:

\[ t_2 = \frac{l}{\left| v_A - v_B \right|} \]  

The concentrations of A and B formed during this time are:

\[ A = B < Ck_t \]  

The maximum change in concentration of A (or B), ΔA, due to the recombination during time \( t_2 \) is:

\[ \Delta A = ABk^2t^2 < C^2K_d^{-1}k^3t^3 \]  

Here, we used \( k = k^2K_d^{-1} \). For the assumption of slow recombination to be satisfied, the concentration of recombined A (or B) should be much smaller than the concentration of formed A (or B):

\[ \Delta A \ll A \]  

Using expressions (7)–(9), we can write a stronger condition for negligible recombination:

\[ C^2K_d^{-1}k^3t^3 < Ck_t \]  

Inequality (10) can be simplified:

\[ CK_d^{-1}k^2t^2 < 1 \]  

It is sufficient that inequality (11) is satisfied for the maximum concentration of C, \( C_{\text{max}} \). The maximum concentration of C is the total concentration of the component in deficiency (A or B). Let's define A to be in deficiency, and its total concentration in the equilibrium mixture is \( A_0 \) (including the complex). We can then write:

\[ C_{\text{max}} < A_0 \]  

Using (11) and (12), we can write a stronger condition for slow recombination in phase 2:

\[ A_0K_d^{-1}k^2t^2 < 1 \]  

Finally, using eq 6 and inequality (13), we can define the slow-recombination parameter (SRP) and write the requirement for slow recombination in form:

\[ \text{SRP} = A_0K_d^{-1}k^2l^2\left| v_A - v_B \right|^2 < 1 \]  

Figure 2. NECEEM electropherogram for interaction of AlkB protein and its DNA aptamer.

A less strong condition can be written by a more accurate assessment of \( C_{\text{max}} \) obtained from the quadratic equation for \( C_{\text{max}} \): \( C_{\text{max}}K_d = (A_0 - C_{\text{max}})(B_0 - C_{\text{max}}) \). This will result in a rather large and cumbersome expression yielding two roots, only one of which is the “real” \( C_{\text{max}} \) (the other root will most likely be disqualified as being physically impossible because it will be either negative or greater than \( A_0 \) or \( B_0 \)), and needs to be evaluated on a case by case basis, if at all.

The determination of SRP requires knowledge of the velocity of molecule B which separates from C slower than molecule A does. Molecule B is typically unlabeled, and thus, finding its velocity may require a separate experiment. In the case where A is labeled fluorescently and B does not have such a label, light absorption detection may be used to determine \( v_B \) in a separate experiment. If B is a protein, it can be labeled with Chromo 503 for making B fluorescently detectable without a significant mobility shift. 13

The SRP parameter may be rewritten as \( A_0k^2t^2 \) (using the relation between \( K_d, k \), and \( k_. \)). This allows us to see explicitly that SRP is in fact directly proportional to the rate constant, \( k_+ \), of the recombination reaction as would be expected. It is interesting to note, however, that it is also directly proportional to the rate constant for the dissociation reaction. The latter dependence originates from the influence that \( k_-. \) has on the concentration of the recombining molecules, A and B.

The approaches used to improve the SDP inequality (shortening the plug length \( l \), optimizing the CE conditions) are equally valid for improving the SRP inequality. In addition to these approaches, we also have the benefit of adjusting a third parameter, \( A_0 \). As A is the component in deficiency, it may not be possible to adjust the concentration by any large amount, but a small change in conjunction with a reduction in \( l \) may result in a modest improvement in the SRP.

**Assessing the Slow-Dissociation and Slow-Recombination Assumptions in Experiment.** The two parameters, SDP and SRP, defined above can be easily used to assess whether or not the slow-dissociation and slow-recombination assumptions are satisfied in experiments. Here, we used an interacting pair of the AlkB protein from *E. coli* and its DNA aptamer. 8c The aptamer was labeled fluorescently for sensitive detection. A representative NECEEM electropherogram obtained for this interacting pair is shown in Figure 2. The values of \( k_0 \) and \( K_d \) were calculated using...
simple algebraic equations (see Experimental Section). The migration time of the protein was found using Chromeo 503 labeling as described elsewhere.\(^\text{13}\) The values of the constants, the velocities, and the plug length were used to calculate SDP and SRP. The cumulative results are shown in Table 1.

We also used previously published experimental data to conduct a similar assessment.\(^\text{2,11,12}\) The values of \(A_0\), \(K_D\), \(l\), \(v_A\), \(v_B\), and \(v_C\) were obtained from Materials and Methods section or graphs. In some cases, the values of \(v_B\) were not available and we used \(v_C\) instead. This assumption increased the values of SDP and SRP and thus made requirements 5 and 13 even stronger. Equations 5 and 14 were used to calculate SDP and SRP. These results are summarized in Table 1. Despite a large range of \(A_0\), \(K_D\), \(l\), \(v_A\), \(v_B\), and \(v_C\) values in the analyzed conditions, Equations 5 and 14 were satisfied for all of them: the values of SDP and SRP were well below 1. Thus, all experiments were conducted within the NECEEM assumptions and the determined values of \(k_-\) and \(K_d\) can be trusted.

### Table 1. Examples of Assessing Slow-Dissociation and Slow-Recombination Parameters in NECEEM Experiments

<table>
<thead>
<tr>
<th>molecule</th>
<th>molecule B</th>
<th>(A_0) M</th>
<th>(v_A) cm/s</th>
<th>(v_B) cm/s</th>
<th>(v_C) cm/s</th>
<th>(k_-) s(^{-1})</th>
<th>(K_D) M</th>
<th>(l) cm</th>
<th>SDP</th>
<th>SRP</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA aptamer</td>
<td>MutS protein</td>
<td>(5.0 \times 10^{-8})</td>
<td>0.05</td>
<td>0.10</td>
<td>0.09</td>
<td>(1.7 \times 10^{-3})</td>
<td>0.9</td>
<td>(3.8 \times 10^{-2})</td>
<td>6.8 \times 10^{-4}</td>
<td>ref. 11</td>
<td></td>
</tr>
<tr>
<td>peptide</td>
<td>SH-B(\beta) protein</td>
<td>(1.0 \times 10^{-7})</td>
<td>8.7</td>
<td>12.7</td>
<td>12.7</td>
<td>(9.5 \times 10^{-1})</td>
<td>0.42</td>
<td>(9.9 \times 10^{-2})</td>
<td>3.9 \times 10^{-2}</td>
<td>ref. 12</td>
<td></td>
</tr>
<tr>
<td>15-mer ssDNA</td>
<td>SSB protein(^*)</td>
<td>(1.6 \times 10^{-7})</td>
<td>0.12</td>
<td>0.27</td>
<td>0.27</td>
<td>(3.5 \times 10^{-2})</td>
<td>0.09</td>
<td>(2.1 \times 10^{-2})</td>
<td>2.5 \times 10^{-2}</td>
<td>ref. 2</td>
<td></td>
</tr>
<tr>
<td>15-mer ssDNA</td>
<td>SSB protein</td>
<td>(2.5 \times 10^{-7})</td>
<td>0.08</td>
<td>0.22</td>
<td>0.18</td>
<td>(9.0 \times 10^{-3})</td>
<td>0.64</td>
<td>(6.0 \times 10^{-2})</td>
<td>1.8 \times 10^{-2}</td>
<td>ref. 11</td>
<td></td>
</tr>
<tr>
<td>DNA aptamer</td>
<td>AlkB protein</td>
<td>(5.0 \times 10^{-9})</td>
<td>0.06</td>
<td>0.13</td>
<td>0.08</td>
<td>(2.5 \times 10^{-3})</td>
<td>0.68</td>
<td>(9.7 \times 10^{-2})</td>
<td>1.0 \times 10^{-2}</td>
<td>this work</td>
<td></td>
</tr>
</tbody>
</table>

\(^*\)\(v_B\) is assumed to be equal to \(v_C\); this assumption makes it more difficult for SDP and SRP to satisfy inequalities 5 and 14. \(^b\) Binding parameters are different for two SSB-DNA experiments due to the difference in the buffers used.

### CONCLUDING REMARKS

We introduced a method for easy assessment of whether or not NECEEM can be reliably used for measuring \(k_-\) and \(K_d\) under a certain set of experimental conditions. If the values for both SDP and SRP are considerably less than unity, then NECEEM can be used for accurately measuring \(k_-\) and \(K_d\). SDP and SRP can range between 0 and \(+\infty\). If either of the SDP or SRP values is found not to satisfy this assumption, experimental conditions should be adjusted to reduce the parameter. The simplest condition to adjust is the length, \(l\), of the injected plug of equilibrium mixture. SDP decreases linearly with decreasing \(l\) while SRP decreases quadratically with decreasing \(l\). When decreasing \(l\) is impossible (e.g., due to limitations in detection), the separation conditions can be adjusted to increase the differences between \(v_A\) and \(v_B\) and/or \(v_A\) and \(v_C\). This can be achieved relatively easily by increasing the electric field, as was demonstrated by Kennedy and coauthors.\(^\text{12}\) Precautions should be taken, however, to ensure that the temperature in the capillary is not raised appreciably. Specifically, the temperature in the noncooled capillary inlet can increase significantly with a moderate increase in the field strength. A recently introduced simple method for temperature determination in both cooled and noncooled regions of the capillary will help to avoid sample overheating in NECEEM.\(^\text{14}\) If the values of SDP and SRP are still not much less than unity, then NECEEM is not a suitable method for studying such a reaction and other KCE methods should be considered as an alternative. Macroscopic approach to studying kinetics at equilibrium (MASKE) and equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM) would likely suit such “fast” reactions.

### AUTHOR INFORMATION

Corresponding Author

E-mail: skrylov@yorku.ca.

### ACKNOWLEDGMENT

This work was funded by the Natural Sciences and Engineering Research Council of Canada.

### REFERENCES