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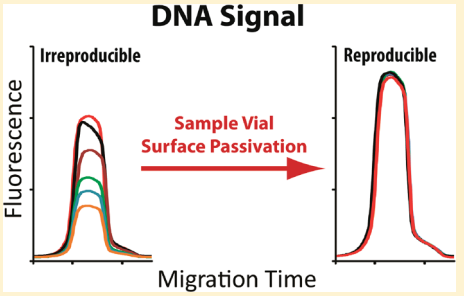
DNA Adsorption to the Reservoir Walls Causing Irreproducibility in Studies of Protein−DNA Interactions by Methods of Kinetic Capillary Electrophoresis

Mirzo Kanoatov and Sergey N. Krylov*

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

ABSTRACT: Methods of kinetic capillary electrophoresis (KCE) facilitate kinetic studies of protein−DNA interactions and highly efficient selection of DNA aptamers for protein targets. Here, we report a previously unnoticed source of error that affects the precision and accuracy of KCE-based measurements. The error manifests itself in cases that require the use of low concentrations of DNA. In such measurements, the reproducibility of the signal generated by the same fluorescently labeled DNA sample can have a relative standard deviation (RSD) as high as 40%. We have investigated the cause of the irreproducibility and found that it is attributed to DNA adsorption to the surface of the sample vials, in which protein−DNA mixtures are prepared prior to a KCE experiment. The use of commercially available "high DNA recovery" sample vials does not resolve the problem. We have found that the problem can be significantly alleviated by the passivation of the vial surface with blocking agents, such as masking DNA or bovine serum albumin (BSA). The described adsorption of DNA to the surface of sample vials may also be important in other procedures that deal with low DNA concentrations, such as aptamer selection and quantitative PCR.



Kinetic capillary electrophoresis (KCE) is a toolkit of homogeneous affinity methods that facilitate precise and accurate measurements of kinetics for intermolecular interactions.[1] The versatility of KCE allows the methods to be customized for virtually any pair of interacting molecules. KCE methods are effective even for some of the more challenging measurements, such as those of very fast association/dissociation rates,[2] complex interactions,[3] or very strong interactions defined by low equilibrium dissociation constants (K_d).[4,5] Other benefits of KCE include low sample consumption and short analysis time. Some of the KCE methods, such as non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM), allow accurate determination of kinetic and equilibrium constants from as few as a single experimental trial.[6] As an electrophoresis-based platform, KCE toolkit is especially well suited for studies of intermolecular interactions of DNA, due to the easily predictable mass-to-charge ratio of oligonucleotides.[7,8] In addition to measurements of affinity interactions, KCE methods are used for highly efficient partitioning in selection of DNA aptamers[9] and facilitate the selection of aptamers with predetermined kinetic and thermodynamic parameters (smart aptamers).[10]

In practice, a KCE user first needs to establish a certain set of initial and boundary conditions for the interacting molecular pair within the capillary. These conditions are determined by the particular KCE method chosen. Mixtures of interacting molecular pairs, or the individual components, are typically prepared in small sample vials and injected into the capillary in a specific order. Electrophoresis is then carried out, during which the various molecular components are allowed to interact. On the basis of the pattern of electrophoretic migration of the molecular components, conclusions can be made about their interaction. For measurements of equilibrium and rate constants, most KCE methods require that the prepared sample mixture contains its molecular components at concentrations comparable to the equilibrium dissociation constant (K_d). This requirement stems from an assumption that the studied molecular pair interacts with a well-defined stoichiometry. If the interacting molecular pair is mixed at a concentration significantly higher than the true K_d of the system, then the measurement of the apparent K_d value will be confounded by molecular crowding effects and interactions of studied molecules at nontypical stoichiometries. While KCE measurements with component concentrations significantly above K_d can be used as means of rough K_d approximation, reduction of component concentrations to levels of apparent K_d is required for a more accurate measurement.[11] This requirement creates a limitation to the lowest K_d value that can be measured by KCE, defined by the sensitivity of the employed instrument. Current commercial instrumentation, equipped with laser-induced fluorescence detection (LIF), offer limits of detection in the low nanomolar to high picomolar range.[12] This range is common for equilibrium dissociation constants of biomolecular interactions, including those of many drugs and inhibitors.[13−15] The use of modern LIF-equipped capillary electrophoresis instrumentation should thus make KCE a perfect platform for studying such interactions. We, however, have recently observed a previously unreported source of random error that significantly

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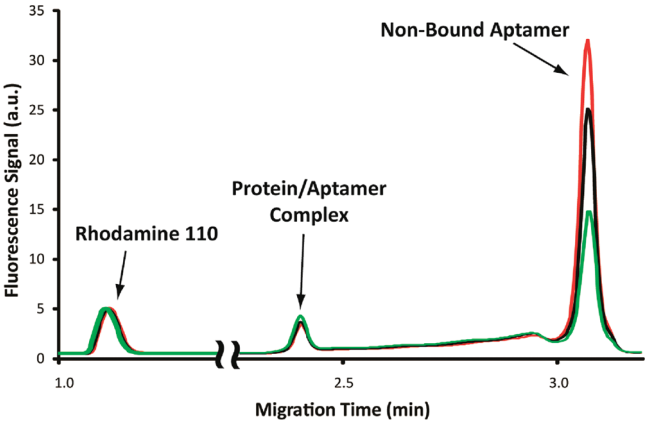

Figure 1. Observed irreproducibility in protein−DNA interaction. All three traces were produced by identical sample mixtures, each containing 35 nM AlkB protein, 25 nM DNA aptamer, and 25 nM rhodamine 110. DNA aptamer and rhodamine 110 were premixed as concentrated stocks and added to the equilibrium mixtures as a single volume.

affects the accuracy and precision of such KCE-based measurements. In cases where low nanomolar (<50 nM) concentrations of DNA are required, the signal produced by the same fluorescently labeled DNA sample can have a relative standard deviation (RSD) of up to 40%. This irreproducibility can greatly effect the results of KCE-based measurements.

To illustrate this issue, we have used the interaction between the *E. coli* protein AlkB and a DNA aptamer. Preliminary affinity tests predict that the K_d value for this interaction is under 50 nM. Thus, for more accurate measurements, equilibrium mixtures containing 35 nM of AlkB and 25 nM of the aptamer were prepared and analyzed using NECEEM. Rhodamine 110 was added to the concentrated stock of the aptamer to act as a signal normalization standard. Signal normalization between electropherograms allowed us to avoid sampling bias due to error in hydrodynamic injection of the sample into the capillary and errors associated with the detection of fluorescence. As seen in Figure 1, signal produced by the nonbound fraction of the DNA aptamer has very poor reproducibility between the three trials. The K_d values calculated from these traces range between 17 nM and 60 nM: an RSD of 50%. Such irregularities in signal response can make KCE methods inaccurate for studies of intermolecular interactions with low nanomolar K_d values. Here, we investigate the cause of the described irreproducibility and propose strategies to eliminate it.

RESULTS AND DISCUSSION

The most intuitive explanation for changes in DNA signal between multiple injections of the same sample is degradation of the sample or photobleaching of its fluorophore. In both of these cases, a clear time-dependent decrease in signal should be observed. To test this hypothesis, a mixture of 25 nM rhodamine 110, 5 nM fluorescein, and 25 nM DNA aptamer was prepared, and multiple injections from the same vial were made. By injecting from the same sample vial multiple times, we were able to avoid the sampling bias associated with pipetting error during sample preparation. Fluorescein was introduced as a third point of reference, to better gauge the relative change between the peaks. As seen in Figure 2A, the DNA aptamer peak shows low reproducibility between trials (RSD of 38%). Furthermore, there is no clear time-dependent decrease in signal intensity, as some of the sample injections produced a higher intensity peak than preceding ones. Evidence of DNA degradation, usually signified by the appearance of new faster-traveling peaks, was also not observed in these electropherograms. These observations suggest that the observed irreproducibility is not due to sample degradation or fluorophore photobleaching. The observed results also eliminate adsorption of DNA to the surface of the bare-silica capillary as the source of the irreproducibility. Adsorption of analytes to the capillary surface manifests itself in "peak tailing", a change in peak width and symmetry. Thus, if the observed reproducibility issues were due to differential adsorption of DNA to the surface of capillary, the observed peaks would differ in the amount of tailing they present. As seen in Figure 2A, this was not the case. Peaks corresponding to DNA had almost identical widths and symmetry and varied only in height. Interestingly, it was noticed that the non-normalized peak intensity of rhodamine 110, a positively charged molecule, varied to a much lesser degree than peaks of negatively charged fluorescein and the DNA aptamer. This observation led us to the idea that the irreproducibility might be caused by electrostatic interactions between DNA and the wall of the sample vial. In this case, higher concentrations of DNA should be affected to a lesser extent by the irreproducibility, as long as the sample volume, and thus the surface area available for interaction, remains constant. As seen in Figure 2B, increasing the concentration of samples by a factor of 20, decreased the RSD of DNA peak intensity to 7%. Furthermore, it was noticed that, at low DNA concentrations, brief vortexing of the sample mixture prior to injection into the capillary increased the intensity of the DNA peak.

These observations are in line with our hypothesis that there is an uneven distribution of DNA molecules within the sample vials due to interaction of the DNA with their surface. In such cases, irregularities in the produced signal may be due to random positioning of the capillary within the sample vial during the sample injection stage of each of the trials. Vortexing of the sample mixture disturbs such interactions and temporarily increases the effective concentration of DNA in the sample, yielding a higher signal.

Unwanted nonspecific interactions of analytes with surfaces of the experimental environment are common in biology and chemistry. The most widespread strategy to eliminate such interactions is to passivate, or block, the surface of the sample vial by an inert molecule. For example, in Southern and Northern blots, the nylon filter is often blocked by nonlabeled, masking DNA, while in Western Blots the nitrocellulose membrane is blocked by bovine serum albumin (BSA) or nonfat milk.[16] To test surface passivation, reproducibility of DNA signal was tested in the presence of an excess of nonlabeled masking DNA (Figure 3). As expected, the RSD of DNA signal intensity in the sample that contained 1 μM masking DNA decreased from 38% to 2%.

In a large number of cases, the use of masking DNA will be an effective strategy for elimination of the described DNA adsorption to the surface of the sample vial. However, the use of masking DNA may not always be suitable. For example, when studying proteins with inherent DNA-binding capabilities, the presence of excess masking DNA will significantly affect its ability to interact with a specific affinity ligand. As an alternative to masking DNA, we have investigated other potential solutions, including the use of other masking agents, such as BSA and heparin. BSA is often used as a surface-blocking agent, albeit to prevent nonspecific protein adsorption, but is also commonly used as a stabilizing

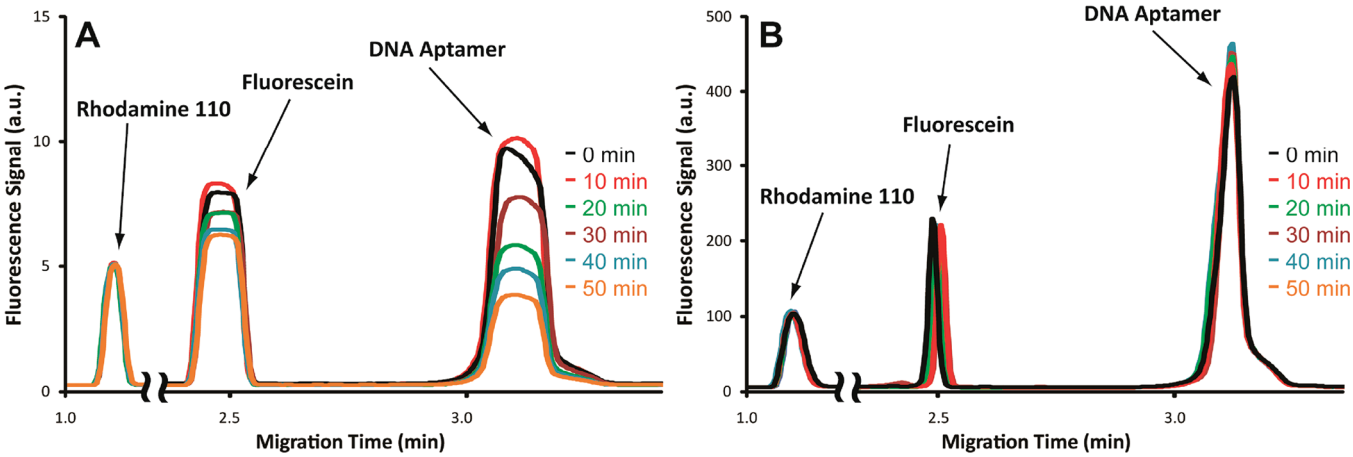

Figure 2. Reproducibility of DNA signal peak intensity at 25 nM concentration (A) and 500 nM concentration (B) of DNA sample. Mixtures also contained 25 and 500 nM rhodamine 110, respectively, and 5 and 100 nM fluorescein, respectively. Graphs were obtained by injecting and analyzing the same mixtures every 10 min. For both cases, the chronological order of the produced electropherograms, starting from the earliest one, is the following: black, red, green, brown, blue, and orange.

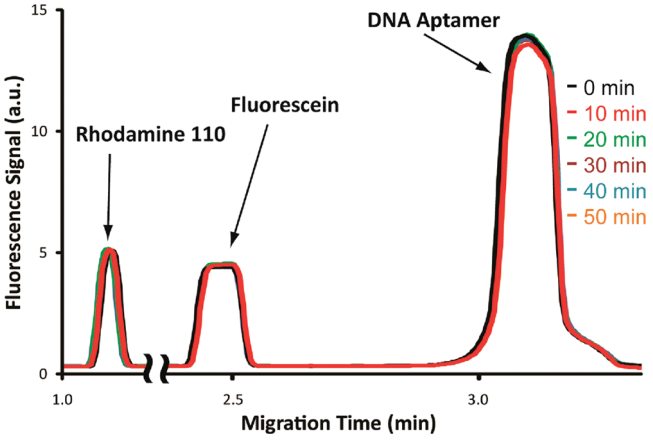

Figure 3. Effects of masking DNA on sample adsorption. Concentration of nonlabeled masking DNA was titrated until RSD of the system was <2%. At this point, the concentration of masking DNA was 1 μM. The chronological order of the produced electropherograms, starting from the earliest one, is the following: black, red, green, brown, blue, and orange.

agent during DNA aptamer selection.[17] Heparin is a molecule that has a similar negative charge density as DNA and is sometimes used to mimic DNA in various applications.[18,19] In addition to surface blocking, we also tested a number of commercially available sample vials that are designed for high DNA recovery.

Similar to the experiment performed with masking DNA, BSA concentration was titrated until RSD of DNA signal intensity was <2%, at which point its concentration was 1 mg/mL. Titration of heparin, unfortunately, did not yield any positive results: at a concentration of 50 μM of heparin, the RSD of the DNA peak intensity marginally decreased from 38 to 32%, while introducing severe changes to patterns of electrophoretic mobility of the analytes. Throughout the described experiments, "Thin Wall PCR tubes" from Axygen were used to make sample mixtures prior to injection into the capillary. We have also investigated the use of sample vials designed for high DNA recovery, including "PCR Maxymum Recovery vials" from Axygen and "DNA LoBind vials" from Eppendorf. Unfortunately, neither of these products provided any improvements to reproducibility, as RSD for both brands remained around 40%.

With masking DNA and BSA showing an ability to reduce DNA adsorption to the sample vial, we tested the effects of these blocking agents on NECEEM measurements. Equilibrium mixtures containing 35 nM of AlkB, 25 nM of the DNA aptamer, and 25 nM of rhodamine 110 were prepared in the presence of each blocking agent. As seen in Figure 4, the reproducibility of nonbound DNA aptamer peak intensity improved significantly in both cases, when compared to Figure 1. As a result, in the presence of masking DNA, K_d was measured at 33 $\pm$ 1 nM (Figure 4A), while in the presence of BSA it was measured at 8 $\pm$ 2 nM (Figure 4B). The relatively high K_d value measured in the presence of the masking DNA can be explained by the fact that AlkB is a DNA-binding protein. As mentioned previously, nonspecific binding of AlkB to an excess of masking DNA can confound affinity measurements of a specific aptamer.

CONCLUDING REMARKS

For KCE methods to be applicable to a wide range of biomolecular systems, reproducible measurements of low concentration samples need to be possible. We described a previously unreported source of error that can significantly affect the precision of such measurements. While additional direct evidence might still be required, the presented observations suggest DNA adsorption to the surface of sample vials as the most likely cause for the described phenomenon. While the two proposed solutions to the issue will be beneficial in a large number of cases, additional strategies for elimination of the DNA adsorption to surface of sample vials might be required. In addition to KCE measurements, the observed source of error may also contribute to other applications where low concentrations of DNA are required. It would be interesting to investigate the effects of the described DNA adsorption in these applications, which include hybridization assays for detection of low abundance DNA sequences, quantitative PCR, and aptamer selection through Non-SELEX.[20] It should noted that the decision to use blocking agents in aptamer selection should be considered with care, as their

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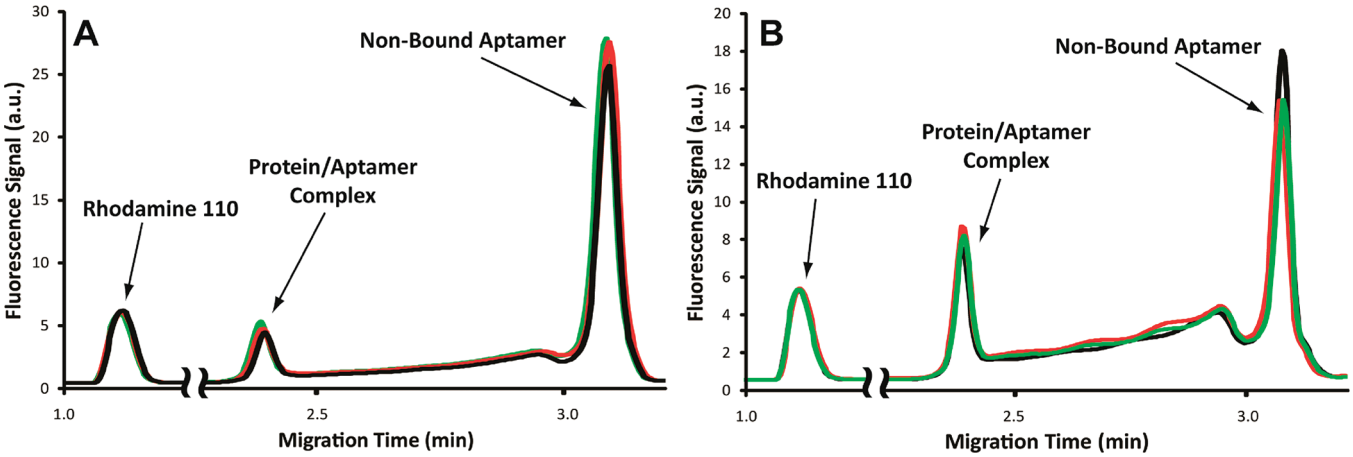

Figure 4. Reproducibility of NECEEM electropherograms in the presence of 1 μM masking DNA (A) and 1 mg/mL BSA (B). Other than the blocking agents, the sample mixtures for all traces were identical to those in Figure 1 and contained 35 nM AlkB protein, 25 nM DNA aptamer, and 25 nM rhodamine 110. DNA aptamer and rhodamine 110 were premixed as concentrated stocks and added to the equilibrium mixtures as a single volume.

presence might influence the interactions between potential aptamer sequences and the target molecule. That being said, both BSA and nonamplifiable background DNA are commonly used in aptamer selection procedures to eliminate nonspecific interactions between naive library molecules and the target. In many cases, their addition did not prevent the selection of high affinity aptamers.[17,21,22] We, thus, foresee that the proposed strategies for elimination of DNA adsorption to surface of vials will be applicable to a wide range of targets for aptamer selection.

MATERIALS AND METHODS

All chemicals and buffer components were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated. Fused-silica capillaries were purchased from Polymicro (Phoenix, AZ). Purified AlkB protein from *E. coli* was kindly donated by Eleanor Bagg and Christopher Schofield from Oxford Centre for Integrative Systems Biology (University of Oxford, UK).[23] DNA aptamer to AlkB was selected as reported previously.[5] DNA sequences were custom synthesized by Integrated DNA Technologies (Coralville, IA). The nucleotide sequence of the fluorescently labeled, single-stranded DNA AlkB aptamer was: 5′-/FAM/-CTCCTCTGACTGTAACCACGTGCCTAGCGTTT-CATTGTCCCTTCTTATTAGGTGATAATAGCATAGGT-AGTCCAGAAGCC-3′. The nucleotide sequence of the nonlabeled, single-stranded masking DNA was: 5′-GGTGGTGGTGGTG-GTG GTG$(T)_{44}$GGTGGGTGGGTGGGTGG-3′.

All capillary electrophoresis (CE) procedures were performed using the following instrumental setup. 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 20 μm and outer diameter of 360 μm were used. Runs were performed in a 30 cm long (20 cm to the detection window) capillary. Twenty five mM Borax at pH 9.2 was used as the electrophoresis run buffer, which was used to fill the inlet and the outlet reservoirs. Prior to every run, the capillary was rinsed with the run buffer solution at 40 psi pressure for 2 min. At the end of each run, the capillary was rinsed with 100 mM HCl, 100 mM NaOH, and deionized water, with the same pressure for 2 min each. The samples were injected into the capillary, prefilled with the run buffer, by a pressure pulse of 28 s at 1 psi. 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 capillary; the direction of the electroosmotic flow was from the inlet to the outlet reservoir. Separation was carried out by an electric field of 830 V/cm. The temperature of the capillary was maintained at 15 °C during the separation.

To observe the reproducibility of the DNA signal intensity, a mixture of 25 nM rhodamine 110, 5 nM fluorescein, and 25 nM DNA aptamer was prepared in a 20 μL volume, and multiple injections were made from the same vial. Unless stated otherwise, "Thin Wall PCR tubes" (Axygen) were used to make sample mixtures prior to injection into the capillary. The sample was stored at 4 °C between injections. In each set of experiments, the sample was injected every 10 min. Electropherograms were normalized by the rhodamine 110 signal. Integrated peak areas were used to calculate the RSD. Subsequently, similar mixtures were also tested in the presence of each: 1 μM masking DNA; 1 mg/mL BSA; 100 μM heparin; as well as using "PCR Maxymum Recovery" (Axygen), "DNA LoBind" (Eppendorf), and generic glass CE vials (Beckman-Coulter).

To obtain the NECEEM experimental data, the interaction between AlkB protein and a DNA aptamer was studied. Equilibrium mixtures were prepared with electrophoresis run buffer and contained 35 nM AlkB protein, 25 nM DNA aptamer, and 25 nM rhodamine 110 (internal standard). The mixture was incubated at 15 °C for 5 min before being injected, to allow equilibration of the components. The DNA aptamer was fluorescently labeled for detection. Some equilibrium mixtures also contained one of the following: 1 μM unlabeled masking DNA or 1 mg/mL BSA. In both cases, equilibration time was increased to 30 min. All samples described in this work, including mixtures of AlkB and its aptamer, were prepared with 25 mM Borax at pH 9.2 as the incubation buffer. Matching of the incubation and electrophoresis run buffers was specifically made to avoid errors associated with sample zone distortions at sample/running buffer interfaces during electrophoresis. Associated peak areas and migration times were obtained from the resulting electropherograms,

and used, as described previously,[24] to calculate equilibrium dissociation constant with the following equation:

$$K_d = \frac{B_0 - A_0\left(1 - \dfrac{S_A}{S_A + S_{C,int} + S_{C,dis}}\right)}{\left(\dfrac{S_A + S_{C,int} + S_{C,dis}}{A_A}\right) - 1}$$

where B_0 and A_0 are initial concentrations of AlkB protein and DNA aptamer after mixing but before the complex is formed, respectively; S_A, $S_{C,int}$, and $S_{C,dis}$ are integrated signal areas of free aptamer peak, AlkB-aptamer complex peak, and exponential decay region, respectively.

AUTHOR INFORMATION

Corresponding Author
*E-mail: skrylov@yorku.ca.

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