# Volatile Kinetic Capillary Electrophoresis for Studies of Protein–Small Molecule Interactions

Jiayin Bao and Sergey N. Krylov*

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

S *Supporting Information*

ABSTRACT: Kinetic capillary electrophoresis (KCE) is a toolset of homogeneous affinity methods for studying kinetics of noncovalent binding. Sensitive KCE measurements are typically done with fluorescence detection and require a fluorescent label on a smaller-sized binding partner. KCE with fluorescence detection is difficult to use for study of protein–small molecule interactions since labeling small molecules is cumbersome and can affect binding. A combination of KCE with mass-spectrometry (KCE-MS) has been recently suggested for label-free studies of protein–small molecule interactions. The major obstacle for studies by KCE-MS is a buffer mismatch between KCE and MS; MS requires volatile buffers while KCE of protein–ligand interactions is always run in near-physiological buffers. Here we asked a simple question: can protein–ligand interactions be studied with KCE in a volatile buffer? We compared three volatile buffers (ammonium acetate, ammonium bicarbonate, and ammonium formate) with a near-physiological buffer (Tris-acetate) for three protein–ligand pairs. The volatile buffers were found not to significantly affect protein–ligand complex stability; moreover, when used as CE run buffers, they facilitated good-quality separation of free ligands from the protein–ligand complexes. The use of volatile buffers instead of Tris-acetate in detection of small molecules by MS improved the detection limit by approximately 2 orders of magnitude. These findings prove the principle of "volatile" KCE, which can be easily coupled with MS to facilitate label-free kinetic studies of protein–small molecule interactions.

NECEEM electropherograms
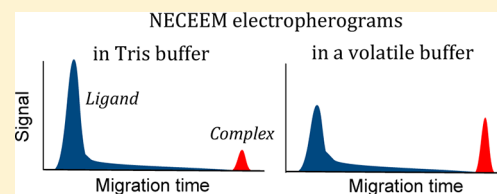
Protein–small molecule affinity interactions play an important role in regulatory biological processes.[1] Furthermore, the action of most prospective small-molecule drugs is based on drugs' ability to form affinity complexes with their therapeutic targets, which are typically proteins.[2] The formation and dissociation of an affinity complex, P•SM, between protein, P, and small molecule, SM, are characterized by rate constants k_{on} and k_{off} of the forward and reverse processes, respectively:

$$P + SM \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} P \bullet SM$$
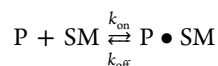
and the stability of the complex is described in terms of the equilibrium dissociation constant $K_d = k_{off}/k_{on}$. Since the three constants, k_{on}, k_{off}, and K_d, are interconnected, determining any pair of constants will define the third.

Kinetic affinity methods can measure k_{on}, k_{off}, and K_d for protein–small molecule binding and are, thus, essential for understanding the dynamics of biological processes and developing protein-binding small-molecule drugs.[3] Such methods fall into two major categories: heterogeneous and homogeneous.[4] Heterogeneous methods require the immobilization of a small molecule on the surface of a sensor for sensitive detection.[5] The immobilization of a small molecule is usually difficult without affecting its ability to bind the protein.[6] Homogeneous methods do not require the immobilization of any of the binding partners and are, in general, preferred over heterogeneous methods in protein–small molecule studies.[7]

However, homogeneous methods often require labeling of a small molecule, which reduces advantages over heterogeneous methods.

Kinetic capillary electrophoresis (KCE) is a toolset of homogeneous kinetic affinity methods with a potential application to protein–small molecule studies.[8] Conceptually, the protein and small molecule are allowed to interact; then, the protein–small molecule complex is separated from the small molecule by capillary electrophoresis, and the small molecule is detected at the end of the capillary (the small molecule is chosen for detection as it experiences a much greater mobility shift than the protein within the affinity complex). The values of k_{on}, k_{off}, and K_d are then determined from the temporal propagation pattern (signal versus migration time) of the small molecule. Most KCE applications have been developed with fluorescence detection to allow high sensitivity and selectivity.[9] Since the small molecule is detected in KCE of protein–small molecule interactions, it should be labeled for fluorescence detection. Fluorescent labeling of small molecules is impractical in most cases.

As an alternative to KCE with fluorescence detection, we have recently suggested KCE with mass-spectrometry (MS) detection which can facilitate label-free analysis of protein–small molecule binding.[10] A similar approach has also been

implemented by Sun and coauthors.[11] Our experiments revealed a serious obstacle in the way of making KCE-MS a widely used practical tool. Following the "tradition" of affinity methods, KCE has been always run in near-physiological buffers, such as Tris-acetate and Tris-HCl, when protein–ligand interactions were studied. These buffers are not suitable for MS as they suppress ionization and, thus, lead to a poor limit of detection (LOD). As an illustration, we compared the LOD for electrospray ionization MS of alprenolol dissolved in a nonvolatile Tris-acetate and volatile ammonium acetate and found it to be approximately 30 nM and 300 pM, respectively. It is clear that Tris-acetate cannot be used in KCE-MS of highly stable complexes with low nM affinity as such studies need to be conducted at small molecule concentrations below the LOD. In addition, Tris-acetate leads to solid deposition at the ionization source, which requires its impractically frequent cleaning. We have, thus, been developing approaches to resolve the problem of buffer mismatch in KCE-MS.

While investigating relatively sophisticated modifications to MS, we asked ourselves a very simple question: Can volatile buffers that are suitable for MS be used for KCE involving proteins? On the one hand, we could not find in the literature references to the use of volatile buffers in affinity studies involving proteins. On the other hand, we could not find any reference to the incompatibility of protein–ligand interactions with "non-toxic" volatile buffers. We, thus, set a goal to test a few volatile buffers in KCE analysis of a few protein–ligand pairs. We chose three nontoxic volatile buffers that can be used at neutral pH values: ammonium acetate, ammonium bicarbonate, and ammonium formate. Tris-acetate was used as a near-physiological buffer control. The three protein–ligand pairs investigated were single-stranded DNA binding (SSB) protein with single-stranded DNA (ssDNA), α_1-acid glycoprotein (AGP) protein with bodipy, and MutS protein with its DNA aptamer. Both DNA molecules were fluorescently labeled for detection; bodipy is a fluorescent dye that requires no labeling.

A KCE method termed nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM)[12] was used in this work because it allowed us to separately study the influence of buffers on protein–ligand complex stability and on separation of unbound ligand from protein–ligand complex. In NECEEM, an equilibrium mixture of the interactants is first prepared in an incubation buffer; a small volume of mixture is sampled for electrophoresis, and the protein–ligand complex is separated from the unbound ligand in a run buffer. The values of K_d and k_{off} can be determined from peak areas in an electropherogram. Equilibrium is established in the incubation buffer and, thus, K_d corresponds to the incubation buffer conditions. Complex dissociation, in contrast, occurs in the run buffer; thus, k_{off} is measured under the conditions of the separation buffer. The two buffers may be different or the same.

■ EXPERIMENTAL SECTION

Chemicals and Materials. Human AGP protein, fluorescein, and all buffer components were obtained from Sigma-Aldrich (Oakville, ON, Canada). Fluorescently labeled ssDNA oligonucleotides 5′-CCC TAT AGT GAG TCG TAT TA-3′ and MutS aptamer 5′-CTT CTG CCC GCC TCC TTC CTG GTA AAG TCA TTA ATA GGT GTG GGG TGC CGG GCA TTT CGG AGA CGA GAT AGG CGG ACA CT-3′ were purchased from IDT DNA Technology Inc. (Coravile, IA, USA). SSB protein from *Escherichia coli* was ordered from

Epicentre Biotechnologies (Madison, WI, USA). The UltraTrol dynamic coating was purchased from Target Discovery, Inc. (Palo Alto, CA, USA). MutS protein was purchased from GenScript (Piscataway, NJ, USA). Bodipy was purchased from Invitrogen (Grand Island, NY, USA). The fused-silica capillaries were purchased from Polymicro (Phoenix, AZ, USA). All solutions were made using deionized water filtered through a 0.22 μm filter (Millipore, Nepan, ON, Canada).

Instrumentation. All CE experiments were carried out with an MDQ-PACE instrument (Beckman-Coulter, ON, Canada) equipped with a laser induced fluorescent (LIF) detector. All data were recorded with a 4 Hz acquisition rate. The inner and outer diameters of the capillary were 50 and 360 μm, respectively. The total capillary length was 38.5 cm with 28.5 cm from the injection end to the detection window.

Kinetic Capillary Electrophoresis (KCE). NECEEM was used as the model KCE method. Uncoated fused-silica capillaries were used in SSB-ssDNA and AGP-bodipy experiments. The UltraTrol coated capillaries were used in MutS–aptamer experiments. Electrophoresis was run in a 30 kV negative polarity for the coated capillary and 30 kV positive polarity for the uncoated capillary. The nonvolatile buffer was 25 mM Tris-acetate, pH 7.2. There are three volatile buffers: 30 mM ammonium acetate, pH 7.2, 30 mM ammonium bicarbonate, pH 7.8, and 30 mM ammonium formate, pH 7.2. The pre-equilibrated protein–DNA binding mixtures were prepared by mixing 100 nM protein with 100 nM DNA and 100 nM fluorescein as internal standard. The AGP-bodipy mixture was made from 20 μM AGP, 20 μM bodipy with 1 μM fluorescein internal standard. Each control sample contains only ligand (DNA or bodipy) with internal standard. The samples were injected into capillary by a pressure of 0.5 psi for 10 s. Electrophoresis was carried out with a capillary coolant temperature set at 15 °C. All experiments were performed in triplicates.

■ RESULTS AND DISCUSSION

First, we studied the influence of volatile buffers on complex stabilities. The interacting pairs were incubated in volatile buffers and NECEEM was run in Tris-acetate buffer for all volatile incubation buffers. We found that NECEEM electropherograms for volatile incubation buffers were similar to the electropherograms for Tris-acetate incubation buffer. The data for the MutS–aptamer pair are shown in Figure 1, and the data for the other protein–ligand systems are in the Supporting Information. These results suggested that volatile buffers did not significantly change complex stabilities of the three studied protein–ligand pairs.

We then examined how the volatile buffers affected separation of unbound ligands from protein–ligand complexes. In these experiments, the run buffers were identical to the incubation buffers. We found that free ligands could be separated from protein–ligand complexes in all volatile buffers tested. The electropherograms for the MutS–aptamer pair are shown in Figure 2, and those for the other two interacting pairs are in the Supporting Information. Separation was the worst in ammonium acetate; it was the best in ammonium bicarbonate (better than in Tris-acetate). Moreover, peak shapes of the DNA aptamer in ammonium bicarbonate and ammonium formate differed from classical Gaussian (Figure 2, panels C and D). It is known that DNA aptamer can fold into various secondary structures under different conditions, such as buffer composition, pH, and temperature.[13] Peak shape irregularity is
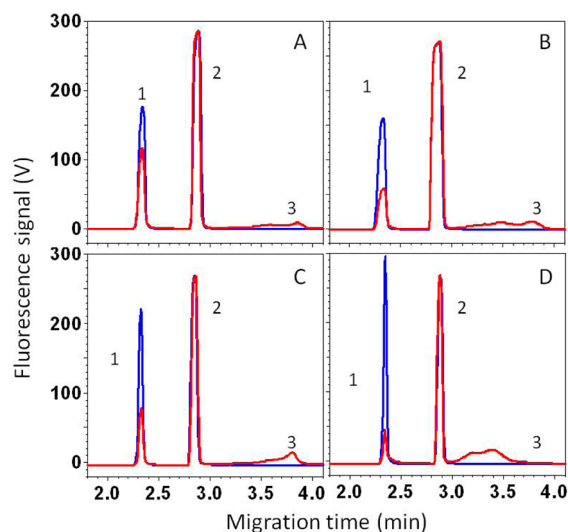
Figure 1. NECEEM electropherograms of MutS−aptamer binding analysis under various incubation conditions: 25 mM Tris-acetate, pH 7.2 (A), 30 mM ammonium acetate, pH 7.2 (B), 30 mM ammonium bicarbonate, pH 7.8 (C), and 30 mM ammonium formate, pH 7.2 (D). The nonvolatile 25 mM Tris-acetate, pH 7.2, was used as separation buffer. Blue and red traces represent the control and binding, respectively. The numbers 1, 2, and 3 indicate the signal of free aptamer, internal standard, and MutS−aptamer binding complex, respectively.
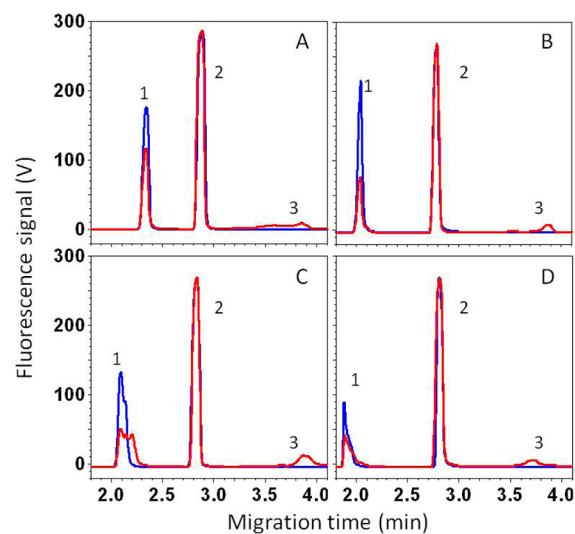

Figure 2. NECEEM electropherograms of MutS−aptamer binding analysis by volatile incubation and separation buffers: 25 mM Tris-acetate, pH 7.2 (A), 30 mM ammonium acetate, pH 7.2 (B), 30 mM ammonium bicarbonate, pH 7.8 (C), and 30 mM ammonium formate, pH 7.2 (D). Blue and red traces represent the control and binding, respectively. The numbers 1, 2, and 3 indicate the signal of free aptamer, internal standard, and MutS−aptamer binding complex correspondingly.

likely caused by the formation of multiple secondary structures of the aptamer in these two buffers. We also found that different buffers could lead to different binding stoichiometries of the affinity complexes (Figure S2 in the Supporting Information). SSB protein is a homotetramer, which can bind to more than one ssDNA molecule.[14] Two peaks corresponding to the SSB−DNA complex are seen in the electropherograms, and the ratio between the peaks changes from buffer to

buffer. The peak with the shortest migration time most likely corresponds to the complex of SSB with a single DNA molecule. The slower migrating complex is likely SSB with two ssDNA molecules.

Finally, we have determined K_d values for all three interacting pairs in the four buffers studied (Table 1). While K_d was found

Table 1. Equilibrium Dissociation Constants (K_d) for Three Pairs of Noncovalent Protein−Ligand Complexes Measured by NECEEM in Three Different Incubation/Run Buffers[a]

buffer	K_d (nM) MutS−aptamer	K_d (μM) AGP-bodipy	K_d (nM) SSB−DNA
Tris-acetate (control)	47 ± 7[b]	5.8 ± 0.8	1.5 ± 0.3
ammonium-acetate	30 ± 3	12.4 ± 0.5	0.5 ± 0.3
ammonium-bicarbonate	43 ± 8	17.9 ± 3.3	7.6 ± 6.5
ammonium-formate	39 ± 9	3.3 ± 2.5	<0.5

[a]See Supporting Information for relevant electropherograms. [b]Mean values $\pm$ one standard deviation ($n = 3$).

to depend on the buffer (which was expected), most values were of the same order of magnitude. The most noticeable effect was that of the ammonium format of SSB−DNA complex: complex stability improved to the level at which K_d was too small to determine its value accurately. The quantitative results unambiguously suggest that volatile buffers did not drastically change complex stability of the three studied protein−ligand complexes.

CONCLUSIONS

The sole goal of this study was to test if volatile buffers could be used for KCE of protein−ligand interactions. This test required that we answered two questions: (1) whether or not volatile buffers significantly affect reversible protein−ligand binding and (2) whether or not volatile buffers can facilitate efficient separation of the protein−ligand complexes from the unbound ligands. These questions could be answered with fluorescently labeled ligands and simple fluorescence detection without relatively sophisticated KCE-MS schemes. Our study strongly suggests that the bias against the use of volatile buffers in studies of protein−ligand interactions is not justified. Specifically, volatile buffers can be used in KCE and will, therefore, facilitate simple coupling of KCE with MS. The tandem of volatile KCE with MS constitutes a homogeneous label-free method that promises to significantly simplify kinetic studies of protein−small molecule interactions.

ASSOCIATED CONTENT

S Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: skrylov@yorku.ca.

Notes

The authors declare no competing financial interest.

6946

dx.doi.org/10.1021/ac301829t | Anal. Chem. 2012, 84, 6944−6947

ACKNOWLEDGMENTS

REFERENCES

(1) (a) Firestone, A. J.; Chen, J. K. *ACS Chem. Biol.* **2010**, *5*, 15−34. (b) He; Ding, Y.; Li, D.; Lin, B. *Electrophoresis* **2004**, *25*, 697−711. (c) Fenteany, G.; Zhu, S. *Curr. Top. Med. Chem.* **2003**, *3*, 593−616.

(2) (a) Imming, P.; Sinning, C.; Meyer, A. *Nat. Rev. Drug Discovery* **2006**, *5*, 821−834. (b) Backes, A. C.; Zech, B.; Felber, B.; Klebl, B.; Müller, G. *Expert Opin. Drug Discovery* **2008**, *3*, 1409−1425.

(3) Swinney, D. C. *Drug Discovery* **2010**, *7*, 53−57.

(4) Newton, P.; Harrison, P.; Clulow, S. *J. Biomol. Screening* **2008**, *13*, 674−682.

(5) (a) Abdiche, Y.; Malashock, D.; Pinkerton, A.; Pons, J. *Anal. Biochem.* **2008**, *377*, 209−217. (b) Rich, R. L.; Myszka, D. G. *J. Mol. Recognit.* **2011**, *24*, 892−914.

(6) Shiau, A. K.; Massari, M. E.; Ozbal, C. C. *Comb. Chem. High Throughput Screening* **2008**, *11*, 231−237.

(7) Jamieson, E. R.; Jacobson, M. P.; Barnes, C. M.; Chow, C. S.; Lippard, S. J. *J. Biol. Chem.* **1999**, *274*, 12346−12354.

(8) (a) Drabovich, A. P.; Berezovski, M. V.; Musheev, M. U.; Krylov, S. N. *Anal. Chem.* **2009**, *81*, 490−494. (b) Bao, J.; Krylova, S. M.; Reinstein, O.; Johnson, P. E.; Krylov, S. N. *Anal. Chem.* **2011**, *83*, 8387−8390. (c) Kim, S. E.; Su, W.; Cho, M.; Lee, Y.; Choe, W. E. *Anal. Biochem.* **2012**, *424*, 12−20. (d) Sloat, A. L.; Roger, M. G.; Lin, X.; Ferrance, J. P.; Landers, J. P.; Colyer, C. L. *Electrophoresis* **2008**, *29*, 3446−3455.

(9) Petrov, A.; Okhonin, V.; Berezovski, M.; Krylov, S. N. *J. Am. Chem. Soc.* **2005**, *127*, 17104−17110.

(10) Bao, J.; Krylova, S. M.; Wilson, D. J.; Reinstein, O.; Johnson, P. E.; Krylov, S. N. *ChemBioChem* **2011**, *12*, 2551−2554.

(11) Sun, J.; He, B.; Liu, Q.; Ruan, T.; Jiang, G. *Talanta* **2012**, *93*, 239−244.

(12) Krylov, S. N. *J. Biomol. Screening* **2006**, *11*, 115−122.

(13) Neves, M. A. D.; Reinstein, O.; Saad, M.; Johnson, P. E. *Biophys. Chem.* **2010**, *153*, 9−16.

(14) Lohman, T. M.; Ferrari, M. E. *Annu. Rev. Biochem.* **1994**, *63*, 527−570.

"Volatile" Kinetic Capillary Electrophoresis for Studies of Protein-Small Molecule Interactions
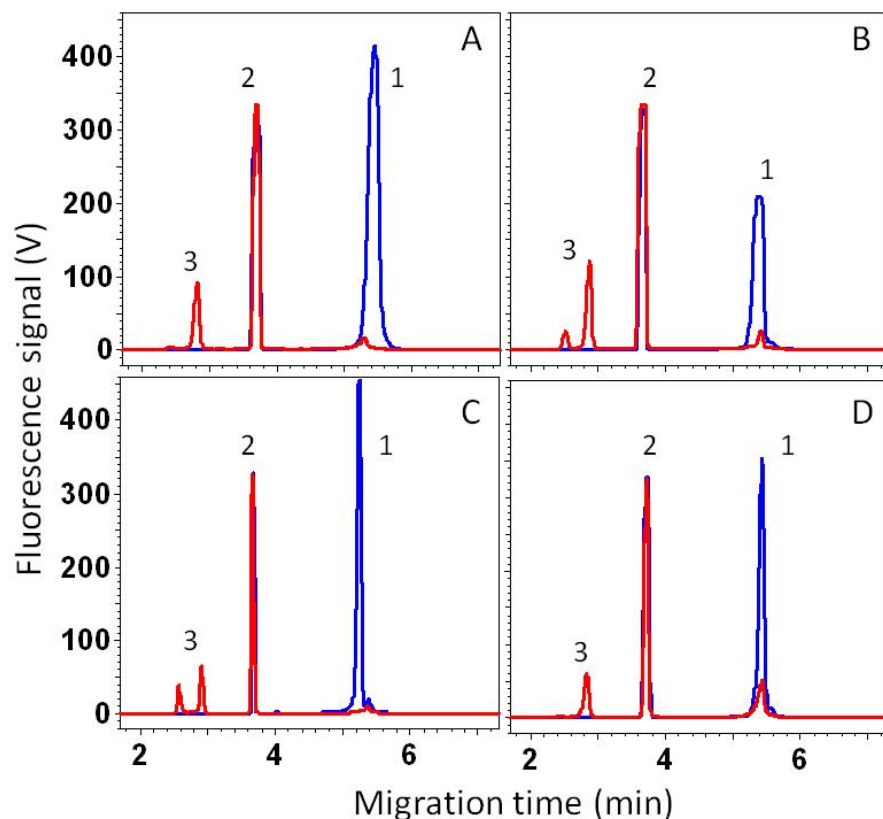
Jiayin Bao and Sergey N. Krylov

Figure S1. NECEEM electropherograms of SSB-ssDNA binding analysis under various incubation conditions: 25 mM tris-acetate pH 7.2 (A), 30 mM ammonium acetate pH 7.2 (B), 30 mM ammonium bicarbonate pH 7.8 (C), and 30 mM ammonium formate pH 7.2 (D). A non-volatile 25 mM tris-acetate pH 7.2 buffer was used as a run buffer. Blue and red traces represent control (no protein, ligand only) and binding experiment (protein plus ligsnd), respectively. The numbers 1, 2, and 3 indicate peaks corresponding to unbound ssDNA, internal standard, and SSB-ssDNA complex, respectively.
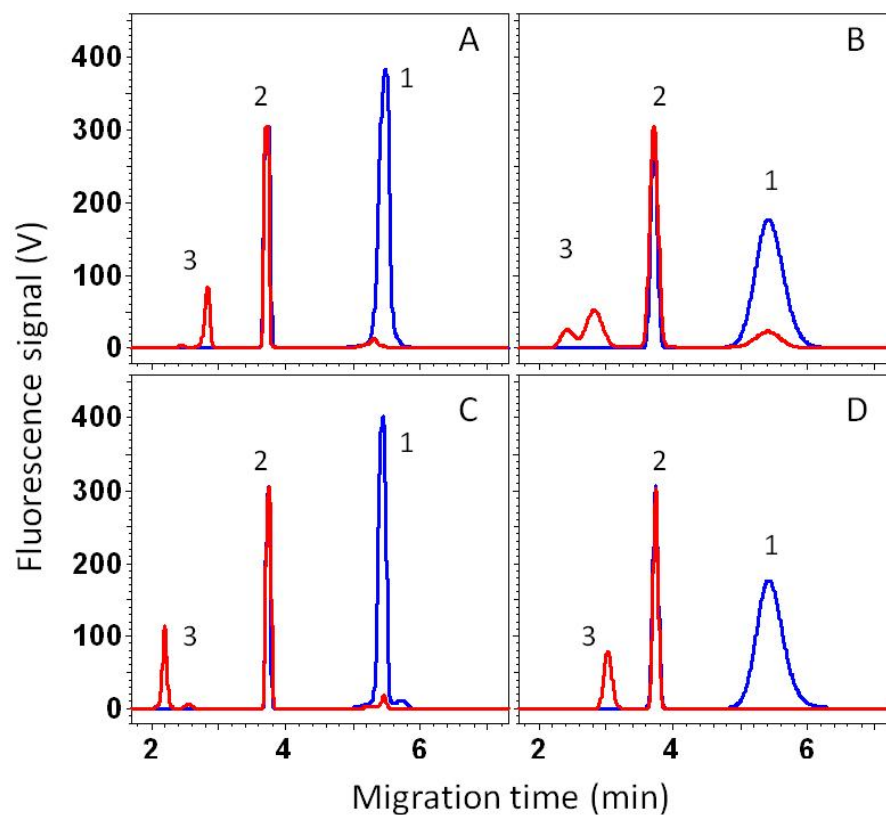
Figure S2. NECEEM electropherograms for SSB-ssDNA interaction studied in the following incubation/run buffers: 25 mM tris-acetate pH7.2 (A), 30 mM ammonium acetate pH 7.2 (B), 30 mM ammonium bicarbonate pH 7.8 (C), and 30 mM ammonium formate pH 7.2 (D). Blue and red traces represent control (no protein, ligand only) and binding experiment (protein plus ligsnd), respectively. The numbers 1, 2, and 3 indicate peaks corresponding to unbound ssDNA, internal standard, and SSB-ssDNA complex, respectively.
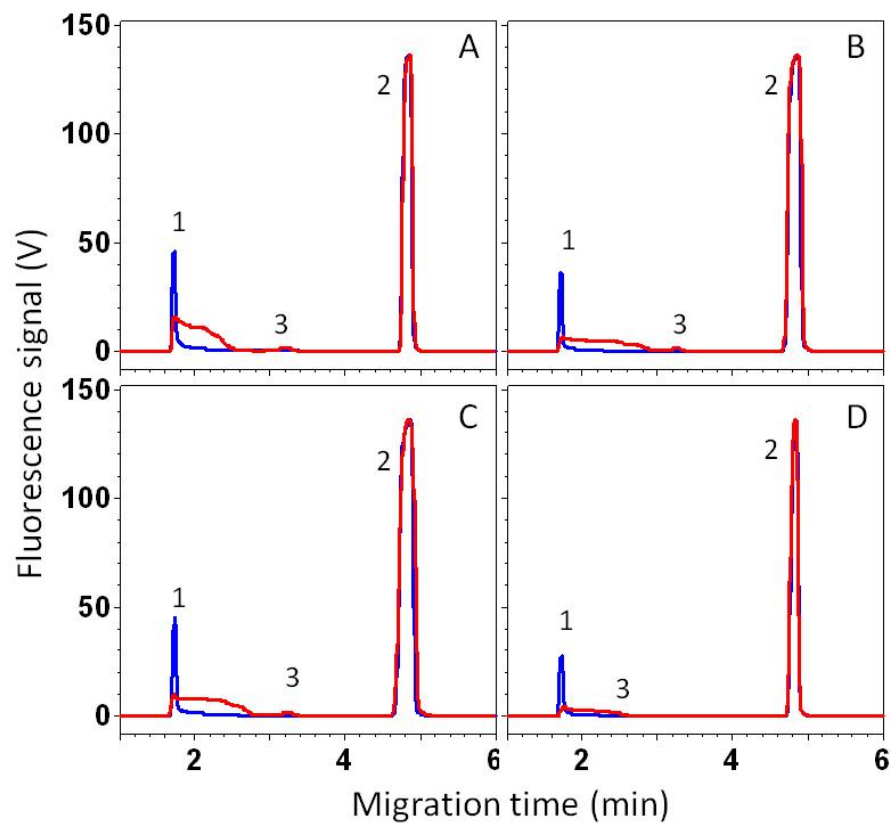
Figure S3. NECEEM electropherograms of AGP-bodipy interaction under various incubation conditions: 25 mM tris-acetate pH 7.2 (A), 30 mM ammonium acetate pH 7.2 (B), 30 mM ammonium bicarbonate pH 7.8 (C), and 30 mM ammonium formate pH 7.2 (D). A non-volatile 25 mM tris-acetate pH 7.2 was used as a run buffer. Blue and red traces represent control (no protein, ligand only) and binding experiment (protein plus ligsnd), respectively. The numbers 1, 2, and 3 indicate peaks corresponding to unbound bodipy, internal standard and AGP-bodipy complex, respectively .
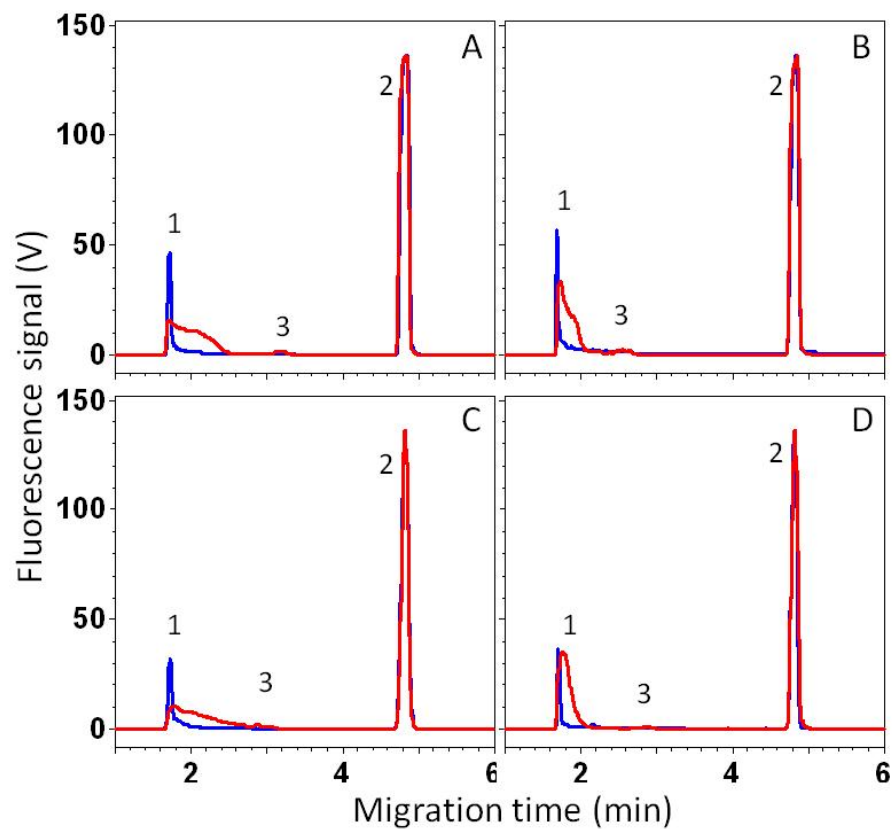
Figure S4. NECEEM electropherograms of AGP-bodipy interaction studied in the following incubation/run buffers: 25 mM tris-acetate pH7.2 (A), 30 mM ammonium acetate pH 7.2 (B), 30 mM ammonium bicarbonate pH 7.8 (C), and 30 mM ammonium formate pH 7.2 (D). Blue and red traces represent control (no protein, ligand only) and binding experiment (protein plus ligsnd), respectively. The numbers 1, 2, and 3 indicate peaks corresponding to unbound bodipy, internal standard, and AGP-bodipy complex, respectively.