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LETTER

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Label-Free Solution-Based Kinetic Study of Aptamer–Small Molecule Interactions by Kinetic Capillary Electrophoresis with UV Detection Revealing How Kinetics Control Equilibrium

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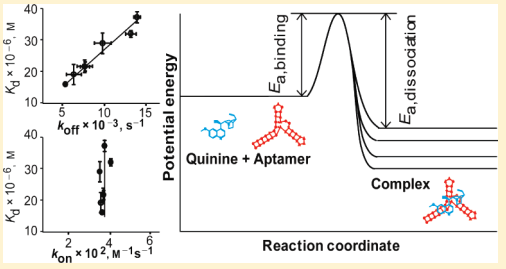
Supporting Information

ABSTRACT: Here we demonstrate a label-free solution-based approach for studying the kinetics of biopolymer–small molecule interactions. The approach utilizes kinetic capillary electrophoresis (KCE) separation and UV light absorption detection of the unlabeled small molecule. In this proof-of-concept work, we applied KCE-UV to study kinetics of interaction between a small molecule and a DNA aptamer. From the kinetic analysis of a series of aptamers, we found that dissociation rather than binding controls the stability of the complex. Because of its label-free features and generic nature, KCE-UV promises to become a practical tool for challenging kinetic studies of biopolymer–small molecule interactions.



DNA and RNA aptamers can be developed to bind different types of targets selectively and with high affinity.[1] In particular, they can be developed for small molecule targets.[2] Such aptamers can facilitate highly sensitive analyses of small molecule toxins, pollutants, biomarkers, etc.[3] Knowing the kinetic parameters of aptamer–small molecule interactions is important for understanding its mechanism as well as for developing practical affinity assays.[4] The kinetics of the interactions is controlled by two parameters: the bimolecular rate constant of binding, k_{on}, and a monomolecular rate constant of complex dissociation, k_{off}:

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} C, \quad K_d = k_{off}/k_{on} \qquad (1)$$

where A and B are interacting molecules and C is the binary complex. Currently available methods for measuring k_{on} and k_{off} of affinity interactions are either label-based, such as stopped-flow fluorescent spectroscopy,[5] or surface-based, such as surface plasmon resonance (SPR).[6] In order to be sensitive to aptamer–small molecule interactions, these methods require that the small molecule be either labeled (typically with a fluorophore) or immobilized on the sensor surface.[7] Both the labeling and the immobilization require small molecule modifications that are difficult to do and that can significantly affect k_{on} and k_{off}.[8] In principle, these assays can be performed with labeling or immobilizing the aptamer instead of the small molecule,[9] but such a format has much lower sensitivity. Here we introduce the first label-free solution-based approach suitable for studying kinetics of aptamer–small molecule interactions. The approach is based on kinetic capillary electrophoresis (KCE)[10] with detection of an unmodified small molecule by UV light absorption. We name this approach KCE-UV. In this proof-of-principle work, we used KCE-UV to study the kinetics and equilibrium for the interaction of variants of a three-way junction DNA aptamer[11] with quinine. The validity of KCE-UV data was confirmed by comparing $K_d = k_{off}/k_{on}$ measured by KCE-UV with K_d measured by isothermal titration calorimetry (ITC).

Our results for seven sequence variants showed that K_d was proportional to k_{off} and did not depend on k_{on} and that k_{on} did not change significantly for different aptamers.

While in this proof-of-principle work we only demonstrate the application of our method to DNA aptamers, it will also be applicable to other aptamers, e.g., RNA or PNA, as they exhibit electrophoretic properties similar to those of DNA aptamers. KCE-UV can also be potentially adapted to study other interacting molecules, such as protein–small molecule. Moreover, other means of detection, such as intrinsic fluorescence or mass spectrometry, can be potentially used for label-free KCE.

In KCE methods, two molecules interact with each other within a capillary while being separated by electrophoresis. The rate constants are determined by analyzing the propagation profile of one of the molecules (typically the smaller one).[10] KCE methods do not require the immobilization of molecules on the surface; moreover, KCE methods do not inherently require labeling. However, until now all KCE studies have been performed with the fluorescent labeling of a smaller molecule.[12] For KCE to be label-free, it should utilize detection of a signal generated by intrinsic properties of the molecule, such as light absorption, native fluorescence, mass signature in mass spectrometry, etc. While the idea of such label-free KCE is not

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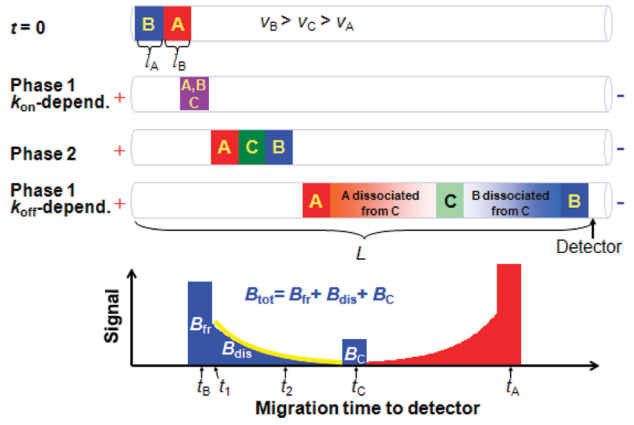

Figure 1. Schematic illustration of the ppKCE method. See the text for details.

counterintuitive, it has never been implemented. The goal of this work is to demonstrate the use of this method for the analysis of aptamer−small molecule interactions.

We chose UV light absorption as a very generic, accessible, and practical means of detection: most molecules absorb light in UV and all commercial CE instruments are equipped with UV absorption detection. The KCE method of choice is plug−plug kinetic capillary electrophoresis (ppKCE),[13] which facilitates direct measurements of k_{on} and k_{off} by first mixing and reacting molecules and then separating and dissociating the formed complex. The concept of ppKCE is schematically presented in Figure 1. At time zero, short plugs of A and B with concentrations of [A] and [B] and length of l_A and l_B, respectively, are sequentially injected one after another by a low-pressure pulse; the components with a lower mobility (e.g., A) is injected first. An electric field is then applied and A and B undergo micromixing due to their differential mobility.[14] When passing through each other, A and B form a certain amount of C which is dependent on [A], [B], k_{on}, and time of passage. When the zones of A and/or B are separated from that of C, C starts dissociating (dissociation fitting is illustrated by the yellow curve) with a rate dependent on k_{off}. A temporal propagation pattern of one component, for example B, is recorded by a detector placed at a distance of L from the capillary inlet. The values of k_{off} and k_{on} can finally be determined by using the signal intensities of B and corresponding migration times from a ppKCE electropherogram:

$$k_{off} = \log\left(\frac{I_{t_2}}{I_{t_1}}\right)\frac{(t_1 - t_C)}{t_C(t_2 - t_1)} \quad (2)$$

$$k_{on} = \varepsilon L(1/t_B - 1/t_A)/([A]l_A) \quad (3)$$

Here I_{t1} and I_{t2} are signal intensities of B at times t_1 and t_2, respectively, in the exponential region of the trace (shown by the yellow line in Figure 1, bottom curve). The migration times of A, B, and C are represented by t_A, t_B, and t_C, where $t_A = t_C$ since the complex comigrates with the aptamers. Parameter ε is determined by solving the following nonexplicit equation:

$$B_{fr}/B_{tot} = \ln\{(\exp(\varepsilon) - 1)\exp(-\varepsilon([B]l_B/([A]l_A))) + 1\}/\varepsilon \quad (4)$$

In the above equations, B_{fr} is the area of peak corresponding to unbound (free) B, B_{tot} is the area of the peak corresponding to total B in the absence of an aptamer (Figure 2a). l_A and l_B are the injection plug lengths of A and B. The other parameters have been defined above. The kinetic rate constant of complex dissociation, k_{off}, can be determined by fitting the experimental decay curve with the exponential dissociation calculation in eq 2.

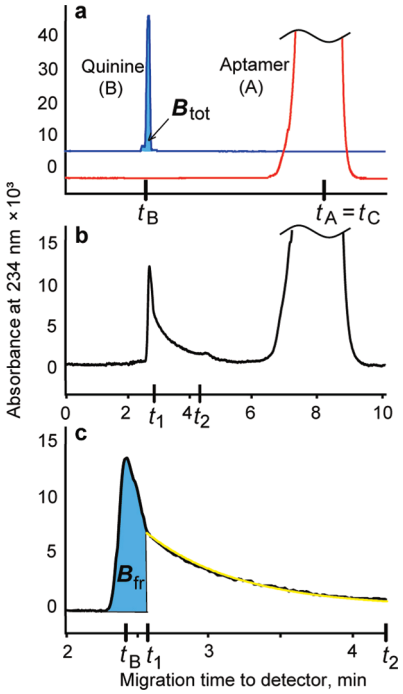

Figure 2. UV detection of quinine and aptamer in capillary electrophoresis: injected separately (a); in a ppKCE experiment (b); enlarged signal for quinine dissociation observation (c). The yellow curve in part c represents the fitted data for the k_{off} calculation. See the Supporting Information for experimental details.

Table 1. Rate Constants of Dissociation (k_{off}) and Binding (k_{on}) for the Interaction of Quinine with Variants of a DNA Aptamer Measured by ppKCE-UV[a]

aptamer name	k_{off} ($\times 10^{-3}$ s^{-1})	k_{on} ($\times 10^{2}$ $M^{-1}s^{-1}$)	K_d (μM)
MN15	5.2 ± 0.1	3.5 ± 0.1	14.8 ± 0.5
MN16	7.6 ± 0.9	3.6 ± 0.1	21.1 ± 2.2
JB3	7.6 ± 0.5	3.6 ± 0.1	20.9 ± 1.6
JB5	6.2 ± 0.9	3.5 ± 0.2	18.2 ± 3.5
JB6	14.0 ± 0.4	3.7 ± 0.1	38.0 ± 1.9
JB8	9.8 ± 1.0	3.4 ± 0.1	29.0 ± 3.6
JB10	13.2 ± 0.7	4.1 ± 0.1	32.3 ± 1.2

[a] See the Supporting Information for sequences and putative secondary structures of the aptamers.

As a molecular model for this study, we chose the interaction between a three-way junction DNA aptamer originally selected to bind cocaine and quinine (a nonregulated alkaloid molecule).[15] All experiments were performed with 25 mM tris-acetate buffer pH 8.2 (see the Supporting Information for experimental details). We define A as the aptamer and B is quinine. Quinine migrates faster than an aptamer (Figure 2a); therefore, in ppKCE-UV experiments, a plug of aptamer solution (123 nL, 150 μM) was injected first, followed by a plug of quinine solution (123 nL, 150 μM). An electric field of 500 V/cm was then applied. Quinine

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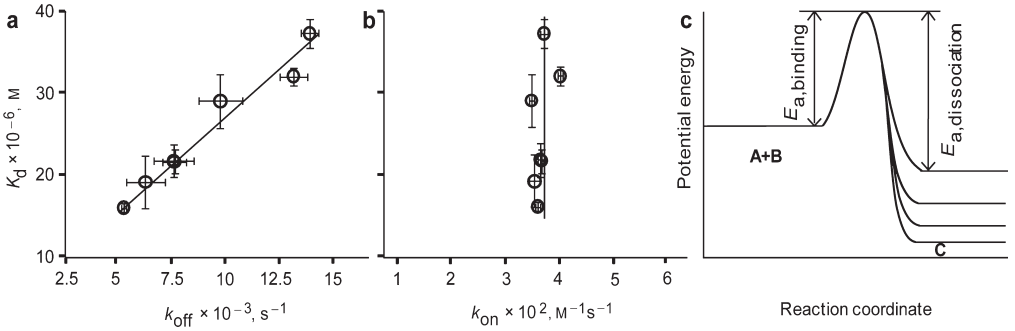

Figure 3. Dependence of K_d (= k_{off}/k_{on}) on k_{off} (a) and k_{on} (b) and schematic energy diagram (c) for the binding of quinine to a set of aptamer variants.

is much smaller than the aptamer and is expected to experience a significant mobility shift upon binding to the aptamer. In contrast, the aptamer mobility should not change significantly upon binding quinine (this was confirmed in ppKCE experiments by observing an exponential dissociation region for quinine but not for the aptamer as shown in Figure 2b). Hence, the propagation pattern of quinine is much more informative than that of the aptamer and quinine was followed in our ppKCE-UV experiments.

The kinetic traces of quinine−aptamer interaction were monitored by UV absorbance of quinine at 234 nm. The wavelength of UV detection depends on the structure of the small molecule studied and also run buffer composition. If the molecule has a chromophore, longer wavelengths can potentially be used for detection.

Seven variants of the original aptamer were synthesized and studied (see Figure S1 in the Supporting Information for sequences and putative secondary structures of the aptamers). We conducted ppKCE-UV experiments for quinine interaction with each mutant in triplicates, at 15 °C with the same buffer (25 mM tris-acetate buffer pH 8.2). Propagation patterns are shown in Figure S2 in the Supporting Information. The values of k_{off}, k_{on}, and K_d were calculated by using eqs 2, 3, and 1, respectively. The results are summarized in Table 1.

To validate our results, we tested K_d values from three aptamer−quinine binding pairs by using isothermal titration calorimetry ITC. ITC is a label-free method which is commonly used to obtain an equilibrium dissociation constant K_d. All ITC experiments were performed in triplicates in a buffer identical to that used in KCE-UV experiments (see a representative thermogram in Figure S3 in the Supporting Information). The K_d values measured with ITC were 14.9 ± 2.7 μM for MN15, 17.5 ± 3.2 μM for MN16, and 21.4 ± 3.1 μM for JB3. The ITC measurements suggest that K_d calculated as k_{off}/k_{on} obtained by KCE-UV were correct. We rely on this validation method since there are no other label-free kinetic methods available for such a validation. Although there is still a possibility that k_{on} and k_{off} are determined with the same systematic error which is canceled upon their division, such an error is very unlikely as the parameters enter equations for k_{on} and k_{off} in different ways.

Finally, we analyzed the data for potential correlation between the kinetic (k_{on} and k_{off}) and equilibrium (K_d) constants. Figure 3a,b shows dependencies of K_d versus k_{on} and K_d versus k_{off}. As evident from the graphs, K_d is linearly proportional to k_{off} (R = 0.96) and does not depend on k_{on} (R = 0.04). This suggests that activation energies are similar for the binding process and vary for the dissociation process (Figure 3c).

The fact that k_{off} changes in a larger range than k_{on} has been noticed by Zhang and coauthors,[16] when they analyzed the kinetic data from a number of oligonucleotide−small molecule binding studies.[17] Prakash analyzed literature data for protein−protein interactions and noticed a similar behavior for some protein−protein pairs.[18]

To conclude, we outline the main features of KCE-UV. KCE-UV is a generic label-free solution-based approach for studying kinetics of affinity interactions. KCE-UV will be especially useful in studies of interactions between biopolymers (e.g., aptamers or proteins) and small molecules. Advantageously, the kinetic parameters are determined based on the propagation pattern of the unlabeled small molecule. In addition, KCE-UV does not require that absorption spectra of the small molecule and the aptamer do not overlap. The method only requires that the small molecule and the aptamer are separated in capillary electrophoresis. Most non nucleic-acid ligands can be well separated from nucleic-acid aptamers and the method is suitable for them. Because of the difficulty of separating nucleic-acid ligands from nucleic-acid aptamer, the method is hardly applicable to such interacting pairs. In this proof-of-principle work, we used ppKCE, one of the simplest KCE methods. In contrast to some other KCE methods, ppKCE is a purely kinetic method as it does not require reaching equilibrium. This feature alleviates a well-known limitation of UV absorption, a relatively poor limit of detection. The problem is overcome by simply using high concentrations of the interacting molecules (the concentrations can be much higher than a K_d value) which is especially useful for small molecules with weak light absorption. The ability of KCE-UV to measure kinetics in a label-free solution-based format along with the simplicity of this approach and the availability of suitable commercial instrumentation makes it attractive to analytical chemists, biochemists, and drug developers.

■ ASSOCIATED CONTENT

Supporting Information. Supporting materials and methods and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Ellington, A.; Szostak, J. *Nature* **1990**, *346*, 818–822.

(2) (a) Lee, S. W.; Zhao, L.; Pardi, A.; Xia, T. *Biochemistry* **2010**, *49*, 2943–2951. (b) Robertson, S. A.; Harada, K.; Frankel, A. D.; Wemmer, D. E. *Biochemistry* **2000**, *39*, 946–954. (c) Rieder, U.; Kreutz, C.; Micura, R. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 10804–10809.

(3) Juskowiak, B. *Anal. Bioanal. Chem.* **2011**, *399*, 3157–3176.

(4) (a) Smith, K. D.; Lipchock, S. V.; Ames, T. D.; Wang, J.; Breaker, R. R.; Strobel, S. A. *Nat. Struct. Biol.* **2009**, *16*, 1218–1224. (b) Latham, M. P.; Zimmermann, G. R.; Pardi, A. *J. Am. Chem. Soc.* **2009**, *191*, 5052–5059.

(5) (a) Murugan, R.; Mazumdar, S. *Arch. Biochem. Biophys.* **2006**, *455*, 154–162. (b) Gilbert, S. D.; Stoddard, C. D.; Wise, S. J.; Batey, R. T. *J. Mol. Biol.* **2006**, *359*, 754–768.

(6) (a) Huber, W.; Mueller, F. *Curr. Pharm. Design* **2006**, *12*, 3999–4021. (b) Linman, M. J.; Abbas, A.; Cheng, Q. *Analyst* **2010**, *135*, 2759–2767. (c) Gebhardt, K.; Shokraei, A.; Babaie, E.; Lindqvist, B. H. *Biochemistry* **2000**, *39*, 7255–7265.

(7) (a) Kanoh, N.; Kyo, M.; Inamori, K.; Ando, A.; Asami, A.; Nakao, A.; Osada, H. *Anal. Chem.* **2006**, *78*, 2226–2230. (b) Elenko, M. P.; Szostak, J. W.; van Oijen, A. M. *J. Am. Chem. Soc.* **2009**, *131*, 9866–9867.

(8) Cooper, M. A. *Anal. Bioanal. Chem.* **2003**, *377*, 834–842.

(9) Jing, M.; Bowser, M. T. *Anal. Chim. Acta* **2011**, *686*, 9–18.

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

(11) (a) Stojanovic, M. N.; de Prada, P.; Landry, D. W. *J. Am. Chem. Soc.* **2000**, *122*, 11547–11548. (b) Neves, M. A. D.; Reinstein, O.; Johnson, P. E. *Biochemistry* **2010**, *49*, 8478–8487. (c) Neves, M. A. D.; Reinstein, O.; Saad, M.; Johnson, P. E. *Biophys. Chem.* **2010**, *153*, 9–16.

(12) Krylov, S. N. *Electrophoresis* **2007**, *28*, 69–88.

(13) Okhonin, V.; Petrov, A.; Berezovski, M.; Krylov, S. N. *Anal. Chem.* **2006**, *78*, 4803–4810.

(14) (a) Krylova, S. M.; Okhonin, V.; Krylov, S. N. *J. Sep. Sci.* **2009**, *32*, 742–756. (b) Krylova, S. M.; Okhonin, V.; Evenhuis, C. J.; Krylov, S. N. *TrAC, Trends Anal. Chem.* **2009**, *28*, 987–1010.

(15) Pei, R.; Shen, A.; Olah, M. J.; Stefanovic, D.; Worgall, T.; Stojanovic, M. N. *Chem. Commun.* **2009** (22), 3193–3195.

(16) Zhang, J.; Lau, M. W.; Ferré-D'Amaré, A. R. *Biochemistry* **2010**, *49*, 9123–9131.

(17) (a) Wickiser, J. K.; Cheah, M. T.; Breaker, R. R.; Crothers, D. M. *Biochemistry* **2005**, *44*, 13404–13414. (b) Wickiser, J. K.; Winkler, W. C.; Breaker, R. R.; Crothers, D. M. *Mol. Cell* **2005**, *18*, 49–60.

(18) Prakash, M. K. *J. Am. Chem. Soc.* **2011**, *133*, 9976–9979.