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Pre-equilibration kinetic size-exclusion chromatography with mass spectrometry detection (peKSEC-MS) for label-free solution-based kinetic analysis of protein–small molecule interactions†

Jiayin Bao,[a] Svetlana M. Krylova,[a] Leonid T. Cherney,[a] J. C. Yves Le Blanc,[b] Patrick Pribil,[b] Philip E. Johnson,[a] Derek J. Wilson[a] and Sergey N. Krylov*[a]

Here we introduce pre-equilibration kinetic size-exclusion chromatography with mass-spectrometry detection (peKSEC-MS), which is a label-free solution-based kinetic approach for characterizing non-covalent protein–small molecule interactions. In this method, a protein and a small molecule are mixed outside the column and incubated to approach equilibrium. The equilibrium mixture is then introduced into the SEC column to initiate the dissociation process by separating small molecules from the complex inside the column. A numerical model of a 1-dimensional separation was constructed to simulate mass chromatograms of the small molecule for varying rate constants of binding.

Non-covalent protein–small molecule interactions are critical in regulating various biological processes.[1–3] Additionally, in drug development, the small molecules are often designed to change protein functions through non-covalent binding.[4–6] Knowing the kinetic rate constants of protein–small molecule interactions is essential in understanding cellular processes and the functions of drugs.[7–10] In a schematic sense, we need to know rate constants k_{on} and k_{off} of the following reaction involving a protein (P), a small molecule (SM), and a protein–small molecule complex (P–SM):

$$P + SM \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} P{-}SM \quad (1)$$

The equilibrium dissociation constant can be calculated through $K_d = k_{off}/k_{on}$ (a smaller K_d value indicates higher affinity binding).

The majority of practical methods for measuring kinetic rate constants of protein–small molecule interactions require modification of either P or SM. In general these methods can be categorized into 2 groups: label-based and surface-based. Label-based methods, for example stopped-flow spectroscopy,[11,12] require attachment of a spectroscopically active molecule, such as a fluorophore, onto either P or SM. The surface-based methods, such as surface plasmon resonance (SPR)[13,14] and bio-layer interferometry,[15,16] require immobilization of either P or SM onto an optical sensor. Moreover, the most sensitive mode of detection requires the immobilization or labeling of the small molecule rather than the protein.[17,18] Modifications of small molecules are difficult to achieve, and they can also drastically affect protein–small molecule binding. Therefore, the label-free solution-based kinetic methods are needed for simple and accurate measurements of k_{on} and k_{off}.

Recently we have introduced a solution-based, label-free approach for kinetic analysis of non-covalent protein–small molecule interactions called kinetic size-exclusion chromatography mass spectrometry (KSEC-MS).[19] In KSEC-MS, generic solution-based kinetic separation is realized in a size-exclusion chromatography (SEC) column; label-free detection of SM is done using tandem mass spectrometry (MS/MS). The shape of the resulting chromatogram, signal (proportional to SM concentration) *versus* time, is defined by k_{on} and k_{off}. The values of k_{on} and k_{off} can be determined by finding a suitable mathematical model and fitting the experimental chromatogram with simulated ones while varying k_{on} and k_{off}. The best fit reveals the appropriate values of k_{on} and k_{off}. Plug–plug kinetic size-exclusion chromatography mass spectrometry (ppKSEC-MS) was our first practical implementation of the KSEC-MS concept. In essence, short plugs of SM and P are separately injected into the column; SM is followed by P. In a SEC column, P moves faster, and during the plug of P passing through the plug of SM, the binding reaction occurs and the P–SM complex is formed. When P outruns SM, the continuous dissociation of the complex starts. We developed a 1-dimensional numerical model for simulating a ppKSEC-MS

[a]*Department of Chemistry and Centre for Research on Biomolecular Interactions, York University, Toronto, Ontario M3J 1P3, Canada. E-mail: skrylov@yorku.ca; Tel: +416-736-2100 ext 22345*

[b]*AB Sciex, 71 Four Valley Dr, Vaughan, Ontario L4K 4V8, Canada*

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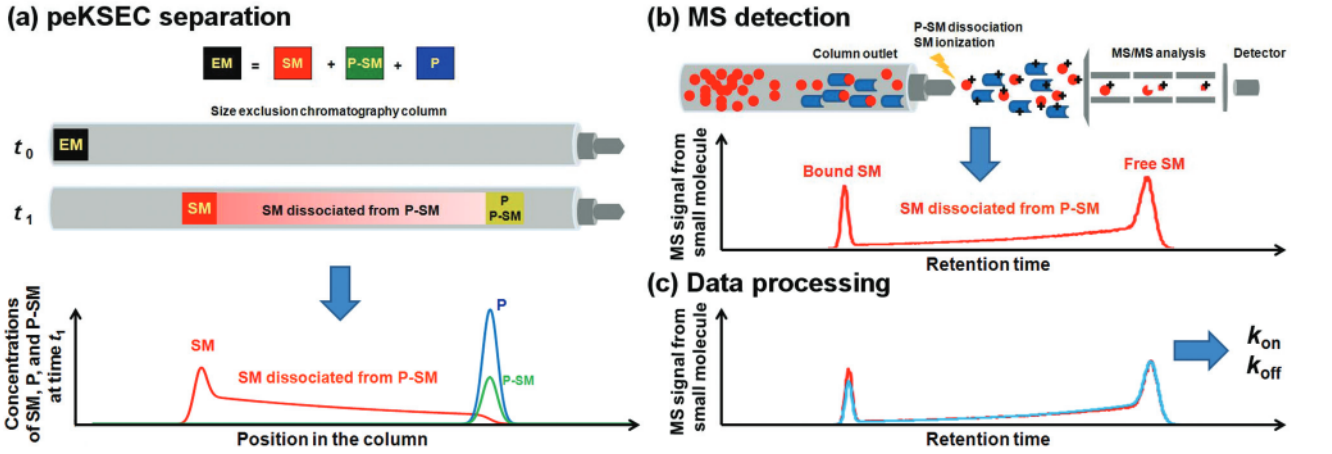

Fig. 1 Conceptual depiction of separation (a), detection (b), and data processing (c) in peKSEC. (a) The EM contains P, SM, and P–SM at equilibrium concentrations. EM is sampled into a SEC column at time t_0. P and P–SM are large and, therefore, move faster than SM and practically together as the size difference between them is negligible. When SM leaves the zone of P–SM, the equilibrium is disrupted and the complex starts dissociating. The newly released SM is continuously separated from P–SM and forms a bridge between the zones of P/P–SM and SM shown at time t_1. The graph illustrates the corresponding concentrations of P, SM, and P–SM at time t_1. (b) The eluate from the SEC column is continuously sampled into a mass spectrometer tuned to detect SM. The P–SM dissociates during ionization to release SM which facilitates indirect detection of the intact P–SM. The resulting KSEC chromatogram contains two peaks and a bridge between them. The fastest peak corresponds to the intact P–SM reaching the end of the column and the slowest one corresponds to SM that was unbound in EM. The bridge corresponds to SM that dissociated from P–SM during the separation. (c) The experimental chromatogram is numerically fitted with a system of partial differential equations describing the processes of chromatographic separation and reaction (1). The values of k_{on} and k_{off} are used as fitting parameters and the best fit corresponds to the sought correct values of k_{on} and k_{off}.

chromatogram, and used it to find k_{on} and k_{off} for the interaction of carbonic anhydrase and its inhibitor, acetazolamide.

To further develop the idea of label-free solution-based kinetic measurements, we now introduce the next KSEC-MS method, pre-equilibration kinetic size-exclusion chromatography (peKSEC). The concept of peKSEC is depicted in Fig. 1. An "equilibrium mixture" (EM) is prepared by incubating P with SM to approach the equilibrium shown in Reaction 1. A small volume of the EM (much smaller than free volume of the column) is injected into a SEC column at time t_0 and its components are separated based on their size differences. As soon as SM is separated from P–SM, the latter is no longer at equilibrium and starts dissociating releasing more unbound SM. The dissociation process continues leaving a "tail" of SM. The samples in the column will eventually elute in the following order: (i) the intact P–SM, (ii) the "tail" of SM that dissociated from the complex during separation, and (iii) the unbound SM in the EM. Upon leaving the column the small molecule can be ionized by various ionization methods, such as electrospray ionization (ESI) or atmospheric-pressure chemical ionization (APCI), and detected.[20] During ionization, the intact P–SM is deliberately destroyed, thus SM from the complex is released and also detected by MS/MS. In general, a peKSEC-MS chromatogram contains 3 features: (i) a peak that corresponds to SM that exited the column as a part of the intact P–SM, (ii) a peak of SM that was unbound in EM, and (iii) a bridge between the two peaks that corresponds to SM that was bound to P but dissociated during separation. The shapes and areas of these three features are defined by k_{on} and k_{off}. Accordingly, fitting the chromatogram with a 1-dimensional mathematical model that describes reaction 1 along with mass transfer in the chromatographic column reveals both rate constants k_{on} and k_{off}.

The great strength of peKSEC-MS is that it relies on a generic separation as a small molecule can always be separated from a large P–SM complex. Moreover, peKSEC-MS uses a generic detection scheme, as practically any small molecule can be selectively detected by MS/MS. Here, we chose the interaction between dihydrofolate reductase (DHFR) and methotrexate (MTX) as a model system. DHFR is an essential enzyme in cell proliferation and cell growth; it converts dihydrofolic acid to tetrahydrofolic acid, and MTX is its well-known inhibitor.[21–23] DHFR and MTX can be easily separated by SEC based on the difference in their sizes. The MS/MS signal intensity of MTX was proportional to the concentration in a range of 10 μM–100 pM; the linear response in the nanomolar range is essential for studying high affinity binding (nanomolar K_d). When EM of DHFR and MTX was sampled for detection of MTX, only a predicted peKSEC-MS chromatogram was obtained in Fig. 2. The signal intensity of the leftmost peak, which corresponds to P–SM increased with increasing protein concentration [P] in EM. A similar trend was also observed for the bridge region, which corresponds to SM that dissociated from P–SM during separation. Meanwhile, as we anticipated, the rightmost peak, free SM, decreased with increasing [P] in EM. The integral of the SM signal over the entire chromatogram remained constant with changing [P] (and the concentration of small molecule [SM] remained constant) suggesting that the intact complex was completely dissociated and all SM was accounted for.

Deconvolution of the kinetic rate constants from a peKSEC-MS chromatogram is not a trivial task. While no analytical

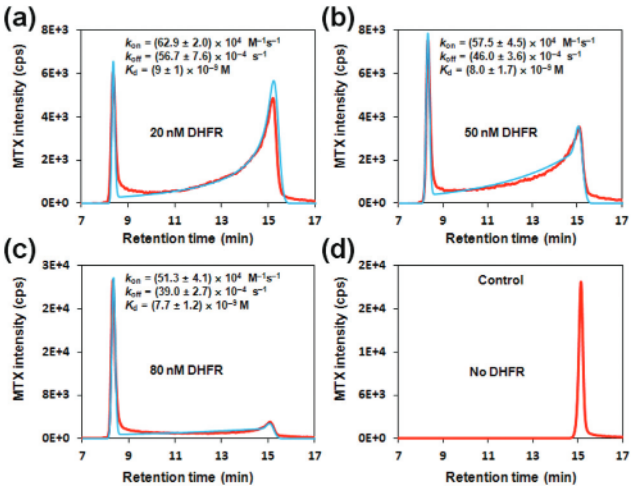

Fig. 2 The peKSEC analysis of DHFR–MTX binding interactions. 20 nM MTX was mixed with different concentrations of DHFR (a)–(c). The control (d) contains MTX only. The MTX signal was detected using MS/MS for 455.2/308.2 (Q1/Q3) *m*/*z*. Simulation chromatograms (blue) were generated from modeling the processes involved in peKSEC by using COMSOL multi-physics software. The kinetic rate constants were determined from the best fit of the experimental chromatogram (red) with the simulated one, and were calculated based on the average and standard deviation of triplicated results.

solutions are available, we have adapted the numerical approach previously developed for modeling ppKSEC-MS to model processes in peKSEC-MS. It is a 1-dimensional model that considers complex dissociation and complex re-formation during migration of the components through the column. The following setup is used for the 1-dimensional approach in peKSEC-MS. A long and narrow cylindrical chromatography column is used, which is coaxial with the x coordinate. It is filled with beads that constitute the stationary phase. The beads have pores which are, in the first approximation, large enough for the SM to enter and reside inside for a significant time and too small for the P or the P–SM to be significantly retarded. This is confirmed by the significant difference in retention times between SM and P–SM. Also, the model uses an assumption of fast re-equilibration between the mobile and stationary phase, which is confirmed by narrow peaks of P and SM. An EM plug containing SM, P, and P–SM is injected into the column at $t = 0$ (t_0). We assume that the buffer velocity and concentrations of components P and P–SM are averaged across the column over the area lying outside the beads. Similarly, the concentrations of SM outside the beads and inside them are averaged across the column over the area lying outside the beads and inside the pores, respectively. Mass transfers of SM, P, and P–SM are described by the following equations:

$$(\partial_t + v_{SM}\partial_x - D_{SM}\partial_x{}^2)[\mathrm{SM}] = \alpha(k_{off}[\mathrm{P\text{–}SM}] - k_{on}[\mathrm{SM}][\mathrm{P}]) \quad (2)$$

$$(\partial_t + v\partial_x - D_P\partial_x{}^2)[\mathrm{P}] = k_{off}[\mathrm{P\text{–}SM}] - k_{on}[\mathrm{SM}][\mathrm{P}] \quad (3)$$

$$(\partial_t + v\partial_x - D_P\partial_x{}^2)[\mathrm{P\text{–}SM}] = k_{on}[\mathrm{SM}][\mathrm{P}] - k_{off}[\mathrm{P\text{–}SM}] \quad (4)$$

$$v_{SM} = \alpha v, \quad \alpha = \frac{\phi_{out}}{\phi_{out} + \phi_{in}}, \quad D_{SM} = \frac{\phi_{out}D_{out} + \phi_{in}D_{in}}{\phi_{out} + \phi_{in}} \quad (5)$$

Here, [SM], [P], and [P–SM] are the concentrations of the small molecule, protein, and the complex, respectively; v is the average velocity of the buffer; D_{out} and D_{in} are diffusion coefficients of SM outside the beads and inside their pores; D_P is the diffusion coefficient of P and P–SM (we consider them similar since SM does not significantly affect the size of P upon binding); ϕ_{out} and ϕ_{in} are relative volumes (*i.e.* fractions of the column volume) located outside beads and inside pores, respectively. Average concentrations of SM outside beads and inside pores are considered to be approximately the same due to fast diffusion equilibration between pores and outside the bead volume. Indeed, we usually have $t_{in} \sim {R_{in}}^2/D_{in}$ where t_{in} is the characteristic time of diffusional relaxation between concentrations of small molecules outside the beads and inside their pores and R_{in} is the characteristic size of the beads. The relationship $t_{in} \sim {R_{in}}^2/D_{in}$ follows from the fact that Einstein's characteristic diffusion length $(D_{in}t)^{1/2}$ should be of the order of the characteristic size of beads, R_{in}, if we calculate this length for the characteristic time of diffusional relaxation $t \sim t_{in}$. At $R_{in} \sim 3$ μm and $D_{in} \sim 10^{-5}$ cm^2 s^{-1} calculations give $t_{in} \sim 0.01$ s. Thus $t_{in} \ll t_{sep} = W/(v - v_A)$ where t_{sep} is the separation time which is usually on the order of a few seconds and W is the plug length. It should be noted that coefficient α depends only on the ratio ϕ_{out}/ϕ_{in} that coincides with the ratio of actual (not relative) volumes located outside beads and inside pores. We have also omitted an additional term proportional to ${t_{in}}^{19}$ in the last expression (5) for D_{SM} since we used the fitting procedure to determine D_{SM}.

To formulate initial conditions for eqn (2)–(5) we take into account that the injection usually satisfies the following conditions: (i) the mixture of SM, P, and P–SM is in equilibrium before the injection; (ii) $t_{inj} \ll t_{eq}$, where t_{inj} is the injection time and $t_{eq} = 1/(k_{on}[\mathrm{P}]_0 + k_{off})$ is the equilibration time; and (iii) $t_{in} \ll t_{inj}$. In this case, the concentrations in the injected plug at $t = 0$ (*i.e.* immediately after injection) are determined by the following relations:

$$[\mathrm{SM}]_0 = \alpha[\mathrm{SM}]_{eq}, [\mathrm{P}]_0 = [\mathrm{P}]_{eq}, [\mathrm{P\text{–}SM}]_0 = [\mathrm{P\text{–}SM}]_{eq} \quad (0 \le x \le W, t = 0) \quad (6)$$

$$W = \frac{V_{inj}}{\pi\phi_{out}R^2}, \quad \phi_{out} = \frac{V_{free}}{\pi R^2 L} \quad (7)$$

Here, $[\mathrm{SM}]_{eq}$, $[\mathrm{P}]_{eq}$, and $[\mathrm{P\text{–}SM}]_{eq}$ are concentrations of SM, P, and P–SM in their equilibrium mixture before injection; W is the plug length after injection; V_{inj} is the volume of injected mixture; V_{free} is the free column volume measured by elution of the protein (in the absence of small molecules); R is the inner radius of the column; and L is the column length. Relations (2)–(7) were used to obtain a numerical solution of the problem and to simulate signal $S(t)$ generated by SM. We assume that the intact P–SM that reaches the end of the column dissociates in the mass-spectrometer and SM produced from this dissociation can be detected. As a result $S(t)$

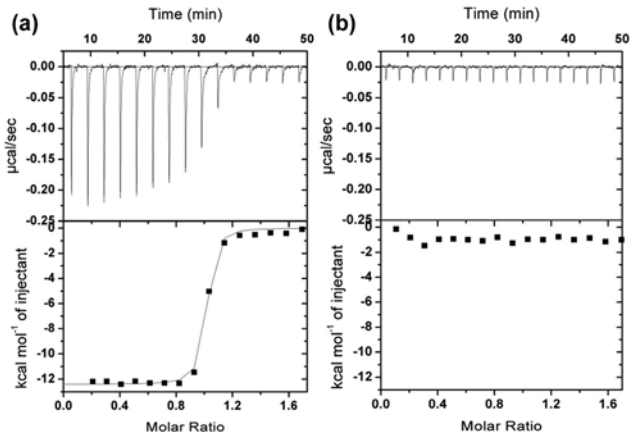

Fig. 3 Thermograms of binding analysis between DHFR and MTX by ITC. (a) Titrating DHFR with MTX and (b) titrating DHFR with buffer only. The upper graphs show the raw ITC data in real time and the lower graphs show the corresponding integrated total heat per injection with respect to molar ratio. The fitting was generated by using Origin 5.0 software with a single-binding-site model.

is proportional to the total concentration of SM (both unbound and bound to P) at the column exit, where g is a proportionality coefficient:

$$S(t) = g([\mathrm{SM}](t) + [\mathrm{P{-}SM}](t)) \quad (8)$$

The model was implemented using COMSOL multi-physics software (4.3a commercial software). The kinetic rate constants k_{on} and k_{off} were convoluted to form a simulated chromatogram. Non-linear regression was used to find the best fit of the experimental peKSEC-MS chromatogram with the simulated one. By fitting the experimental chromatograph to the 1-dimensional model we have calculated the kinetic rate constants: $k_{on} = (57.2 \pm 3.5) \times 10^4\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$, $k_{off} = (47.2 \pm 4.6) \times 10^{-4}\ \mathrm{s}^{-1}$, and $K_d = (8.2 \pm 1.3) \times 10^{-9}$ M through k_{off}/k_{on}. The best fits for different chromatograms (Fig. 2) returned similar values of k_{on} and k_{off} suggesting that the solution is stable and also allowing us to estimate the method's precision. To validate our results, we chose another label free solution based method ITC (Fig. 3). ITC is an equilibrium method from which only K_d can be calculated;[24] it was selected due to the lack of other label-free kinetic methods. ITC and peKSEC-MS are conceptually different methods, offering a means of validation with higher stringency. The ITC measured equilibrium dissociation constant $K_d = (10.2 \pm 0.8) \times 10^{-9}$ M, which agreed with peKSEC-MS measured results within acceptable errors, confirmed the accuracy of peKSEC-MS.

Conclusions

To conclude, we have outlined the main features of peKSEC-MS, which is a label-free solution-based method for studying the kinetics of reversible binding between a protein and a small molecule. In peKSEC, the migration pattern of the small molecule through a SEC column is followed by MS detection, and the kinetic parameters are extracted from the MS signal *versus* time dependence by means of numerical modelling. The numerical model uses two assumptions: (i) complex dissociation is complete during the ionization and (ii) the ionization efficiency of small molecules remains constant. In essence, we assume that the linear response of MS to small molecule concentration is retained throughout the analysis. The requirement of complete dissociation is easily satisfied as it is difficult to keep non-covalent complexes intact during the ionization process. The second assumption may not be always satisfied; for example, the ionization efficiency of a small molecule could be affected when it co-elutes with the protein.[25] Therefore, it is essential to confirm the method's validity by comparing the integrated small molecule signals among all tests – it should remain constant for constant small molecule concentration and not depend on the concentration of the protein. Moreover, measuring fast reactions with low K_d values will require low concentrations of interacting molecules and accordingly lower detection limit of MS.[26–28] The sub-nanomolar K_d measurements will require a mass spectrometer with a sub-nanomolar detection limit. Instrumentation for peKSEC-MS used in this study can measure small molecule concentration of 100 pM and the best contemporary MS instruments have limits of detection in the zeptamolar range.[29] Advantageously, peKSEC-MS does not require MS detection of an intact protein–small molecule complex, which may be very challenging. The ability of MS to rapidly scan through a wide mass range can potentially facilitate simultaneous analysis of one protein with several small molecules that potentially can be used for the rapid screening of panels of drug leads. The peKSEC method allows tight control of binding conditions such as incubation time and temperature, presence of cofactors, *etc*. Furthermore, as peKSEC relies on the established equilibrium prior to injection, we can now analyze the protein–small molecule interactions with slow association rates. Whereas in ppKSEC, the on-column incubation of a slow interacting pair is impractical, and it can also induce sample diffusion and peak broadening. We foresee that peKSEC-MS can become a generic solution-based label-free platform for kinetic studies of protein–small molecule interactions.

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