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Slow-Equilibration Approximation in Kinetic Size Exclusion Chromatography

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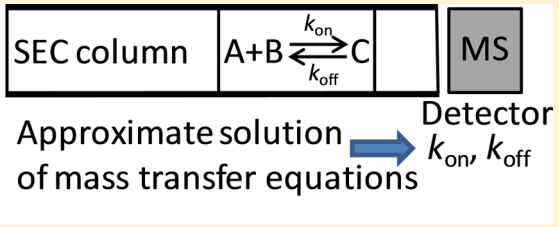
Supporting Information

ABSTRACT: Kinetic size exclusion chromatography with mass spectrometry detection (KSEC-MS) is a solution-based label-free approach for studying kinetics of reversible binding of a small molecule to a protein. Extraction of kinetic data from KSEC-MS chromatograms is greatly complicated by the lack of separation between the protein and protein−small molecule complex. As a result, a sophisticated time-consuming numerical approach was used for the determination of rate constants in the proof-of-principle works on KSEC-MS. Here, we suggest the first non-numerical (analytical) approach for finding rate constants of protein−small molecule interaction from KSEC-MS data. The approach is based on the slow-equilibration approximation, which is applicable to KSEC-MS chromatograms that reveal two peaks. The analysis of errors shows that the slow-equilibration approximation guarantees that the errors in the rate constants are below 20% if the ratio between the characteristic separation and equilibration times does not exceed 0.1. The latter condition can typically be satisfied for specific interactions such as receptor−ligand or protein−drug. The suggested analytical solution equips analytical scientists with a simple and fast tool for processing KSEC-MS data. Moreover, a similar approach can be potentially developed for kinetic analysis of protein−small molecule binding by other kinetic-separation methods such as nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM).



Noncovalent binding of small molecules to proteins plays an important role in regulating many biological processes.[1−3] Modern small molecule drugs are typically designed to change protein functions through binding proteins noncovalently.[4−6] Knowing rate constants, which characterize protein−small molecule interaction, is critical to understanding molecular mechanisms of cellular processes and to the development of highly efficient drugs.[7−10] In essence, we need to know rate constants k_{on} and k_{off} of the following reaction involving a small molecule (A), a protein (B), and a protein−small molecule complex (C):

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} C, \qquad K_d = \frac{k_{off}}{k_{on}} \tag{1}$$

The equilibrium dissociation constant can be calculated from known values of k_{on} and k_{off} using the second relation (1).

The majority of practical methods for measuring k_{on} and k_{off} of protein−small molecule interaction require modification of either the protein or the small molecules. 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 moiety, such as a fluorophore, to either the protein or the small molecule. Surface-based methods, such as surface plasmon resonance (SPR)[13,14] and biolayer interferometry,[15,16] require immobilization of either the protein or the small molecule 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 without drastically affecting k_{on} and k_{off}. Therefore, label-free solution-based kinetic methods are in demand for simple and accurate measurements of k_{on} and k_{off} for protein−small molecule interactions.

Recently, we have introduced a solution-based label-free approach for kinetic analysis of noncovalent protein−small molecule interaction. This approach is kinetic size exclusion chromatography with mass spectrometry detection (KSEC-MS).[19,20] In KSEC-MS, generic solution-based kinetic separation is realized in a size exclusion chromatography (SEC) column, and label-free detection of small molecules is performed with tandem mass spectrometry (MS/MS). The shape of the resulting chromatogram, i.e., signal (proportional to the concentration of small molecules) versus time, is dependent on k_{on} and k_{off}. Two major types of shapes are shown in Figure 1. 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 feasible values of k_{on} and k_{off}. In general, a SEC column is a 3-dimensional separation media, but it can be considered as a 1-dimensional object if the column

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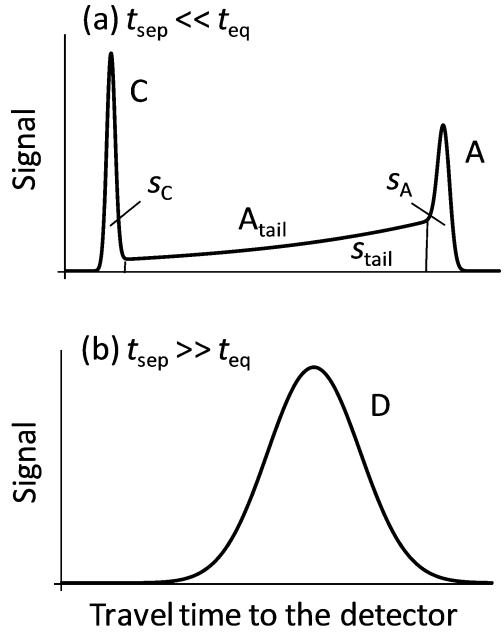

Figure 1. A typical combined signal generated by the small molecule being in both the unbound and protein-bound states in cases of slow (a) and fast (b) equilibration. In the case of slow equilibrium (a), peaks A and C describe intact A and dissociating complex C, respectively, while A_{tail} corresponds to additional A produced by the dissociation of C. In the case of fast equilibrium (b), a wide peak D corresponds to a compound signal from spatially unresolved A and C.

length is much larger (i.e., 1 order of magnitude larger) than the column diameter.

Plug-plug kinetic size exclusion chromatography with MS detection (ppKSEC-MS) was our first practical implementation of the KSEC-MS concept.[19] In this method, a short plug of the small molecule solution is injected into the column first followed by injection of a short plug of the protein solution. In a SEC column, the protein moves faster, and during the protein plug's passing through the small molecule plug, the binding reaction occurs and the small molecule−protein complex is formed. When the protein outruns the small molecule, the continuous dissociation of the complex starts; the dissociation produces a "tail" of the unbound small molecule.

The second practical implementation of the KSEC-MS concept was pre-equilibration kinetic size exclusion chromatography with MS detection (peKSEC-MS).[20] In this method, an "equilibrium mixture" is prepared by incubating the small molecule with the protein to approach equilibrium in Reaction 1. A short plug of the equilibrium mixture is injected into a SEC column and its three components (A, B, and C) are separated on the basis of their size differences. As soon as the small molecule is separated from the complex, the latter is no longer at equilibrium and starts dissociating, releasing the small molecule.

Both ppKSEC-MS and peKSEC-MS assume that $t_{sep} < t_{eq}$ where t_{sep} and t_{eq} are characteristic times of the separation and equilibration processes. In both methods, the three components will eventually elute from the column in the following order: (i) small molecule−protein complex, (ii) the small molecule that dissociated from the complex, and (iii) the small molecule that was unbound in the equilibrium mixture. Upon leaving the column, the small molecule can be ionized by various ionization methods, such as electrospray ionization or atmospheric-pressure chemical ionization, and detected by MS/MS.[20] During the ionization, the intact complex is deliberately destroyed; thus, the small molecule within the complex is freed and also detected by MS/MS.

At $t_{sep} < t_{eq}$, a KSEC-MS chromatogram contains 3 features: (i) a peak that corresponds to the small molecule that exited the column as a part of the complex, (ii) a peak of the unbound initial small molecules, and (iii) a bridge between the two peaks that corresponds to the small molecule that was bound to the protein before the beginning of separation but then 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 k_{on} and k_{off}.

The described above fitting (pattern-based) approach to finding rate constants requires the knowledge of distributions of the small molecule, protein, and complex in the initial plugs. Otherwise, these distributions must be included into a set of the fitted parameters. The use of too many unknown parameters in the fitting procedure could result in lower accuracy of found k_{on} and k_{off}. It may not be clear if the best fit is achieved due to a correct determination of k_{on} and k_{off} or due to an incorrect choice of the unknown distributions in the initial plugs. Besides, the fitting procedure is not mathematically transparent and requires substantial computation time (several hours).

An alternative to the pattern-based approach is a parameter-based approach which relies on finding k_{on} and k_{off} through a small number of characteristic parameters (e.g., areas, widths, heights, and migration times of peaks) determined from experimental KSEC-MS chromatograms. The parameter-based approach has been successfully used for determination of k_{on} and k_{off} by some methods of kinetic capillary electrophoresis.[21−24] Parameter-based methods utilize relatively simple approximate solutions to differential equations for mass transfer. Finding such approximate solutions in KSEC-MS is challenging, but the benefits of a simple parameter-based approach of finding k_{on} and k_{off} in KSEC-MS justifies the effort of finding them. In this work, we report an approximate analytical solution of mass transfer equations in KSEC-MS and a corresponding simple parameter-based method for finding k_{on} and k_{off} for the case of $t_{sep} < t_{eq}$.

RESULTS AND DISCUSSION

One-Dimensional Equations of Mass Transfer in the Presence of a Nonequilibrium Reaction. We consider the application of SEC to study reversible binding of a small molecule (A) and a protein (B) with the formation of their complex (C = AB). We consider the following setup for a 1-dimensional approach in SEC. A long and narrow cylindrical column is coaxial with the x coordinate. A plug containing the equilibrium mixture of A, B, and C is injected in the column at the initial time $t = 0$. The flow of the buffer (mobile phase) and components A, B, and C takes place outside the beads which constitute the size exclusion matrix. Small molecule A can enter bead pores due to diffusion but protein B and complex C are too large for penetrating into the pores. Thus, Reaction 1 can proceed only outside the beads in the free volume of the column. We assume that the buffer velocity and concentrations of components B and C are averaged across the column over the area lying outside the beads. Similarly, the concentrations of A outside the beads and inside them are averaged across the

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column over the area lying outside the beads and inside their pores, respectively.

Mass transfers of A, B, and C are described by the following equations:[25,26]

$$(\phi_{out} + \phi_{in})\partial_t A + v\sigma_{out}\partial_x A - (\sigma_{out}D_{out} + \sigma_{in}D_{in})\partial_x{}^2 A = \phi_{out}(k_{off}C - k_{on}AB) \quad (2)$$

$$\phi_{out}\partial_t B + \sigma_{out}(v\partial_x - D_p\partial_x{}^2)B = \phi_{out}(k_{off}C - k_{on}AB) \quad (3)$$

$$\phi_{out}\partial_t C + \sigma_{out}(v\partial_x - D_p\partial_x{}^2)C = \phi_{out}(k_{on}AB - k_{off}C) \quad (4)$$

where ∂_x and ∂_t are partial derivatives with respect to the spatial coordinate x and time t, respectively; A, B, and C are average concentrations of components A, B, and C, respectively; v is the average velocity of the buffer (mobile phase); D_{out} and D_{in} are the diffusion coefficients of A outside the beads and inside their pores; D_p is the diffusion coefficient of B and C; $\phi_{out} = V_{out}/V$ and $\phi_{in} = V_{in}/V$ are relative volumes outside beads and inside their pores, respectively; $\sigma_{out} = \Sigma_{out}/\Sigma$ and $\sigma_{in} = \Sigma_{in}/\Sigma$ are the relative cross section areas lying outside the beads and inside their pores. Here, V_{out} and V_{in} are volumes outside beads and inside their pores; Σ_{out} and Σ_{in} are cross section areas lying outside the beads and inside their pores; V and Σ are the volume and cross section area of the column. Average concentrations of small molecules outside the beads and inside their pores are considered to be approximately the same due to fast diffusion equilibration. The characteristic time of diffusion relaxation between the concentration of small molecules outside the beads and inside their pores t_{in} can be estimated as follows $t_{in} \sim R_{in}{}^2/D_{in}$, where R_{in} is the radius of beads. For a column with $R_{in} \sim 3$ μm and a small molecule with $D_{in} \sim 10^{-5}$ cm^2/s, we obtain $t_{in} \sim 10^{-2}$ s. On the other hand, the zone separation time $t_{sep} \sim W_0/(v - v_A) > 4$ s for typical values of $W_0 \sim 0.125$ cm, $v \sim 0.06$ cm/s, and $v_A \sim 0.033$ cm/s used in our numerical simulations below. Here, v_A is the observed velocity of the small molecule in the column. Thus, $t_{in}/t_{sep} \sim 2 \times 10^{-3}$ and we can expect that errors resulting from the assumption made (that average concentrations of small molecule outside the beads and inside their pores are approximately the same) will be also ~0.2%.

Diffusion coefficients of the protein and complex are approximately the same (D_p) since their sizes are similar. It is usually assumed in mechanics of multiphase media that the solid phase (i.e., beads) is randomly distributed and $\sigma_{out} = \phi_{out}$ and $\sigma_{in} = \phi_{in}$.[25] As a result, eqs 2−4 can be rewritten as follows:

$$(\partial_t + v_A\partial_x - D_A\partial_x{}^2)A = \alpha(k_{off}C - k_{on}AB) \quad (5)$$

$$(\partial_t + v\partial_x - D_p\partial_x{}^2)B = k_{off}C - k_{on}AB \quad (6)$$

$$(\partial_t + v\partial_x - D_p\partial_x{}^2)C = k_{on}AB - k_{off}C \quad (7)$$

$$v_A = \alpha v, \quad \alpha = \frac{\phi_{out}}{\phi_{out} + \phi_{in}}, \quad D_A = \frac{\phi_{out}D_{out} + \phi_{in}D_{in}}{\phi_{out} + \phi_{in}} \quad (8)$$

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 the beads and inside their pores.

Initial and Boundary Conditions for Eqs 5−7. To formulate initial and boundary conditions for eqs 5−7, we take into account that the injection usually satisfies the following three conditions: (i) the mixture of A, B, and C is in equilibrium immediately before the injection; (ii) $t_{inj} \ll t_{eq}$, where t_{inj} is the injection time and t_{eq} is the equilibration time defined by expression 13 below; (iii) $t_{in} \sim R_{in}{}^2/D_{in} \ll t_{inj}$. In this case, concentrations of A, B, and C in the injected plug at $t = 0$ (i.e., immediately after injection) are determined by relations:

$$A = \alpha A_0, \quad B = B_0, \quad C = C_0; \quad 0 \le x \le W_0, \quad t = 0 \quad (9)$$

$$W_0 = \frac{V_{inj}}{\pi\phi_{out}R^2}, \quad \phi_{out} = \frac{V_{free}}{\pi R^2 L} \quad (10)$$

Here, A_0, B_0, and C_0 are equilibrium concentrations of A, B, and C in their mixture before injection; W_0 is the plug length after injection; V_{inj} is the volume of the injected mixture; V_{free} is the column volume accessible to the protein (it can be estimated by elution of the protein); R is the inner radius of the column; L is the column length. The column volume accessible to the protein, V_{free}, is close to the free volume as the column is chosen so that the pores are smaller than the protein. Eqs 9 and 10 also take into account the fact that components B and C can not penetrate the beads.

Signal Simulation in the Pattern-Based and Parameter-Based Approaches. Equations 5−7 and initial conditions (9) and (10) allow one to obtain a numerical solution of the problem and to model signal $S(t)$ generated by the small molecule at the column exit.[20] We assume that the small molecule is released from the complex C before detection, e.g., during ionization in a mass spectrometer. In this case, S is proportional to the total concentration of the small molecule (both unbound and bound to the protein) at the column exit:

$$S(t) = g_A A(t) + g_C C(t) \quad (11)$$

where g_A and g_C are proportionality coefficients; the ratio $g = g_A/g_C$ has a meaning of relative ionization efficiency of free A with respect to that of A bound to B. Such a theoretical signal can be fitted into experimental data at various values of parameters present in eqs 5−7. Values of k_{on} and k_{off} corresponding to the best fit represent by definition the rate constants determined in the pattern-based approach. The corresponding K_d value is easily calculated using expression 1 for K_d in terms of k_{on} and k_{off}. Actually, parameters v_A, D_A, and g_A can be determined by fitting experimental data obtained for the small molecule alone (i.e., in the absence of the protein). Similarly, parameters v, D_p, and V_{free} can be found by fitting data obtained for the protein without the small molecule. Given v_A and v are determined, parameter α can be calculated using the first relation 8. Finally, the injected volume V_{inj} is usually known from the experimental setup. As a result, only parameters k_{on} and k_{off} have to be varied in the fitting procedure involving experimental data obtained from the mixture of A, B, and C. Such a step-by-step fitting can significantly reduce computational time required for determination of k_{on} and k_{off} in the pattern-based approach. Numerical solutions of eqs 5−10 can be found using COMSOL 4.3a commercial software (COMSOL Group, Palo Alto, CA).[20] Theoretical signals can then be calculated by applying relation 11, and the fitting procedure can be performed.

We also used parameters A_{tot} and B_{tot} that represent the nonequilibrium concentrations of A and B in the initial mixture before formation of C. These parameters are related to the equilibrium concentrations A_0, B_0, and C_0 (present in initial conditions (9)) by the following equations:

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$$A_0B_0 = K_dC_0, \quad A_0 + C_0 = A_{tot}, \quad B_0 + C_0 = B_{tot} \tag{12}$$

An alternative parameter-based approach relies on approximate analytical solutions of eqs 5−7. Such solutions can be obtained in some limiting cases dependent on the characteristic time of small molecule separation, t_{sep}, and the characteristic time of equilibration of Reaction 1, t_{eq}. These times are given by

$$t_{sep} = \frac{W_0}{\nu - \nu_A}, \quad t_{eq} = \frac{1}{k_{on}B_0 + k_{off}}, \quad W_0\Sigma = V_0 \tag{13}$$

where W_0 is the length of the initial plug, Σ is the area of the inner cross section of the column, V_0 is the column volume occupied by the plug (V_0 includes the volume of beads located inside the plug), and B_0 is the characteristic concentration of free protein (i.e., the protein concentration in the initial plug). If we take an excess of B to A in the beginning, then we can assume that B_0 in eq 13 is approximately equal to the total protein concentration B_{tot}.

Equations of Slow-Equilibration Approximation. Given eqs 5−7, we can expect that the initial plug splits into two zones moving with velocities ν and ν_A if $t_{sep} \ll t_{eq}$ (Figure 1a). In the opposite case of $t_{sep} \gg t_{eq}$, there will be only one peak (Figure 1b). The faster zone C mainly contains the dissociating complex C and unbound protein B whereas the slower zone A mainly consists of the intact small molecule (Figure 1a). There is also a small molecule released during the dissociation of C in zone C. Since this small molecule moves slower than zone C, it is left behind zone C as a tail (A_{tail} in Figure 1a). This tail merges into zone A. At $t_{sep} \ll t_{eq}$, we can neglect the reverse association of protein and small molecules (released in decay of C) during the removal of these small molecules from zone C. However, such reverse association can be important at $t_{sep} \sim t_{eq}$ and taking it into account at $t_{sep} \ll t_{eq}$ can reduce errors in determination of k_{on} and k_{off}. The approximate solution obtained below is valid for both $t_{sep} \sim t_{eq}$ and $t_{sep} \ll t_{eq}$ though a pattern shown in Figure 1a might not take place for $t_{sep} \sim t_{eq}$. On the basis of our numerical simulations, this pattern occurs at $t_{sep} \ll t_{eq}$ and it is required for a method of obtaining k_{on} and k_{off} from a slow-equilibration approximation to work with reasonable accuracy. To derive approximate analytical solutions used in the parameter-based approach, we multiply eq 5 by $\phi_{out} + \phi_{in}$ and eqs 6 and 7 by ϕ_{out} and then integrate the obtained relations over zone C. As a result, we have the following equations in this zone with corresponding initial and boundary conditions:

$$\frac{da_C}{dt} = -(\phi_{out} + \phi_{in})(\nu - \nu_A)A_{end}\Sigma - \frac{k_{on}a_Cb_C}{\phi_{out}W_C\Sigma} + k_{off}c_C \tag{14}$$

$$\frac{db_C}{dt} = -\frac{k_{on}a_Cb_C}{\phi_{out}W_C\Sigma} + k_{off}c_C, \quad \frac{dc_C}{dt} = \frac{k_{on}a_Cb_C}{\phi_{out}W_C\Sigma} - k_{off}c_C \tag{15}$$

$$a_C(0) = a_{C0}, \quad b_C(0) = b_{C0}, \quad c_C(0) = c_{C0} \tag{16}$$

Here, d/dt is the derivative in the coordinate system moving with zone C, a_C, b_C, and c_C are total amounts of A, B, and C in zone C, A_{end} is the concentration of A at the end of zone C, W_C is the length of zone C, and a_{C0}, b_{C0}, and c_{C0} are initial values of a_C, b_C, and c_C. We neglected diffusion fluxes at the boundaries of zone C since estimates show that longitudinal Peclet number is very large due to a small diffusion coefficient of $D_P \sim 10^{-7}$ cm^2/s.[27] The derivation of eqs 14 and 15 is identical to that described in the two-peak approximation method elsewhere.[24] Using the expansion of A in the Taylor series inside zone C, a value of A_{end} can be approximately expressed in terms of a_C:[24]

$$A_{end} = \frac{3a_C}{W_C\Sigma(\phi_{out} + \phi_{in})} \tag{17}$$

Mathematical Solution for Slow-Equilibration Approximation. Equations 15 lead to the following conservation law:

$$b_C + c_C = b_{tot} \tag{18}$$

where b_{tot} is the total amount of protein B used in the plug preparation (i.e., present in the injected plug in both free and small molecule-bound states). Expressions 17 and 18 allow one to rewrite eq 14 and the second eq 15 in the form:

$$\frac{da_C}{dt} = -\frac{3a_C(\nu - \nu_A)}{W_C} - \frac{k_{on}a_C(b_{tot} - c_C)}{\phi_{out}W_C\Sigma} + k_{off}c_C \tag{19}$$

$$\frac{dc_C}{dt} = \frac{k_{on}a_C(b_{tot} - c_C)}{\phi_{out}W_C\Sigma} - k_{off}c_C \tag{20}$$

Relations 19 and 20 form a closed system of ordinary differential equations for unknowns a_C and c_C if we approximately assume that $W_C = W_0$ (and, therefore, $W_C\Sigma = V_0$). A system of eqs 19 and 20 is nonlinear and can be solved only numerically. However, this system can be simplified if B is taken in excess to A during the preparation of equilibrium mixture. In this case, $b_{tot} \gg c_C$ and we can neglect the second term in $(b_{tot} - c_C)$ in expressions 19 and 20. After that, eqs 19 and 20 become linear and have the following solution:

$$a_C = \eta N\exp(-\lambda t), \quad c_C = N\exp(-\lambda t) \tag{21}$$

Here, N is a non-negative constant that can be expressed in terms of the initial amounts of a_C and c_C. Coefficients λ and η depend on parameters b_{tot}, k_{on}, k_{off}, W_0, V_0, and $\nu - \nu_A$ and are determined by expressions:

$$\lambda = \Omega - \sqrt{\Omega^2 - \frac{3k_{off}}{t_{sep}}}, \quad \eta = \frac{k_{off} - \lambda}{B_{tot}k_{on}} \tag{22}$$

where we used the following two definitions:

$$\Omega \equiv \frac{1}{2}\left(B_{tot}k_{on} + k_{off} + \frac{3}{t_{sep}}\right), \quad B_{tot} = \frac{b_{tot}}{\phi_{out}V_0} \tag{23}$$

At $b_{tot} \gg c_C$, we can also replace B_0 with B_{tot} in relations 13.

According to the first relation 22, we have $\lambda > 0$. It can be also shown that $\eta \geq 0$, similarly to the case of two peak approximation.[24] Thus, solution 21 satisfies the requirement of non-negativity of both a_C and c_C. Relations 21−23 remain valid at $t_{sep} \ll t_{eq}$, but in this case, expression 22 can be simplified by an expansion in small parameter t_{sep}/t_{eq}:

$$\lambda = k_{off}\left(1 - \frac{t_{sep}}{3t_{eq}}\right), \quad \eta = \frac{t_{sep}k_{off}}{3t_{eq}B_{tot}k_{on}} = \frac{t_{sep}K_d}{3t_{eq}B_{tot}} \tag{24}$$

Given relations 21 and 24, the amount of small molecule present in zone C vanishes at $t_{sep}/t_{eq} \to 0$. This fact confirms our previous prediction that the small molecule is quickly removed from zone C if $t_{sep} \ll t_{eq}$. At $t_{sep} \sim t_{eq}$, relations 22 can

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also be simplified if $k_{off} \ll B_{tot}k_{on} \sim 1/t_{sep}$. In this case, expansions in small parameter $k_{off}/B_{tot}k_{on} = K_d/B_{tot}$ give:

$$\lambda = \frac{3k_{off}}{3 + t_{sep}B_{tot}k_{on}}, \quad \eta = \frac{t_{sep}k_{off}}{3 + t_{sep}B_{tot}k_{on}} \tag{25}$$

Relations 21 with approximate expressions 25 give a solution of a system of differential equations obtained from system (19) and (20) by omitting a derivative dc_C/dt, which is possible at $dc_C/dt \sim k_{off}c_C \ll c_C/t_{sep}$.

Determination of k_{off} and k_{on}. Relations 21−23 can be used to determine k_{off} from measurements of signal S generated by the small molecule. We assume that the small molecule is released from complex C before detection, as it takes place in a mass spectrometer. In this case, S is proportional to the total concentration of the small molecule (both unbound and protein-bound) and is determined by relation 11 at $g = 1$. A simple way to find λ is based on measurements of total signals $s_C(L_1)$ and $s_C(L_2)$ from zone C in the temporal pattern (Figure 1) at two different lengths L_1 and L_2 of the column. In this case, the definition of the total amount c_C and relation 11 yield:

$$c_C = \frac{\phi_{out}\Sigma}{g_C}\int_C S(x)\,dx = \frac{\phi_{out}vs_C\Sigma}{g_C}, \quad s_C = \int_C S(t)\,dt \tag{26}$$

Substitution of this expression (written for two different distances L_1 and L_2) into the second eq 21 yields

$$s_C(L_n) = \frac{g_C}{v}(1+\eta)N\exp\left(-\frac{\lambda L_n}{v}\right), \quad n = 1, 2 \tag{27}$$

Two different L values can be achieved by using SEC columns with the same packing material and diameter but different lengths. The consistency in retention behavior for two different column lengths is not very essential, and moreover, the knowledge of L_1 and L_2 is not necessary. It is only important that that one can measure peak areas and retention times for C in columns with different lengths but with nominally the same diameter and packing material. By resolving a system of algebraic eqs 27 with respect to λ, we obtain

$$\lambda = \frac{1}{t_{L_2} - t_{L_1}}\ln\frac{s_C(L_1)}{s_C(L_2)} \tag{28}$$

were $t_{L_1} = L_1/v$ and $t_{L_2} = L_2/v$ are migration times of peak C to the detector in columns 1 and 2, respectively. After that, k_{off} and k_{on} are determined in terms of λ by expressions:

$$k_{off} = \frac{3\lambda - t_{sep}\lambda^2}{3 - t_{sep}\lambda\left(1 + \frac{B_{tot}}{K_d}\right)}, \quad k_{on} = \frac{k_{off}}{K_d} \tag{29}$$

Here, the first equation follows from relations 22 and 23, whereas the second one results from expression 1 for K_d. Since t_{sep} and B_{tot} are known from the experimental setup, expressions 29 require only the knowledge of K_d in addition to λ.

Determination of K_d. A value of K_d can be determined from a nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM)-like procedure by measuring total signals s_C, s_A, and s_{tail} generated by the small molecule located in zones C and A and in the tail, respectively.[23,28] Note that finding K_d in such a procedure is not affected by reassociation, which can affect the ratio of s_C to s_{tail} but does not influence s_A, and the sum of s_C and s_{tail} that defines K_d according to expressions 31 and 32 below (at $g = 1$). To do that, we take into account injection features used in the derivation of initial conditions (9). As a result, the following relations take place in the injected plug before separation begins:

$$\frac{a_0b_0}{c_0V_{inj}} = K_d, \quad V_0 = \frac{V_{inj}}{\phi_{out}}, \quad a_0 + c_0 = a_{tot}, \quad b_0 + c_0 = b_{tot} \tag{30}$$

Here, a_0, b_0, and c_0 are total amounts of A, B, and C, respectively, in the injected plug; a_{tot} is the total amount of the small molecule A used in the plug preparation (i.e., present in the injected plug in both free and protein-bound states); V_{inj} is the injected volume.

Equations 30 allow K_d to be expressed in the form:[23,28]

$$K_d = \frac{\frac{b_{tot}}{V_{inj}}\left(1 + \frac{a_0}{c_0}\right) - \frac{a_{tot}}{V_{inj}}}{1 + \frac{c_0}{a_0}} \tag{31}$$

Ratios a_{tot}/V_{inj} and b_{tot}/V_{inj} are usually known since they are the initial concentrations of A and B used in the preparation of the equilibrium mixture. Ratio a_0/c_0 can be easily determined from signal S generated by the small molecule, since their conservation law and relation 11 lead to the following expression:

$$\frac{a_0}{c_0} = \frac{a_A}{c_C + a_{tail}} = \frac{s_A}{gs_C + s_{tail}}, \quad g = \frac{g_A}{g_C} \tag{32}$$

Here, the total amount of C in zone C, c_C, can be expressed in terms of signal S (see relations 26). Similarly, the total amounts of A in zone A, a_A, and in the tail, a_{tail}, can be expressed in terms of signal S using the following relations:

$$a_A = \frac{(\phi_{out} + \phi_{in})v_As_A\Sigma}{g_A}, \quad s_A = \int_A S(t)\,dt \tag{33}$$

$$a_{tail} = \frac{(\phi_{out} + \phi_{in})v_As_{tail}\Sigma}{g_A}, \quad s_{tail} = \int_{tail} S(t)\,dt \tag{34}$$

Substituting expressions 26, 33, and 34 into the second ratio 32 and taking into account the first and second relations 8, we obtain the last ratio in (32). After that, the substitution of expression 32 for a_0/c_0 into eq 31 gives a final expression for K_d in terms of experimentally measured quantities:

$$K_d = \frac{\frac{b_{tot}}{V_{inj}}\left(1 + \frac{s_A}{gs_C + s_{tail}}\right) - \frac{a_{tot}}{V_{inj}}}{1 + \frac{gs_C + s_{tail}}{s_A}} = \frac{B_{tot}\left(1 + \frac{s_A}{gs_C + s_{tail}}\right) - A_{tot}}{1 + \frac{gs_C + s_{tail}}{s_A}} \tag{35}$$

A recently developed algorithm for simultaneously finding K_d and protein concentration can be applied to the developed KSEC mathematics in the case of the protein concentration not being known.[29]

Computational Results. To study the accuracy of finding K_d, k_{off}, and k_{on} with the slow-equilibration approximation in KSEC, we need to analyze temporal propagation patterns of a mixture of components A and C participating in reaction 1 at various known values of K_d, k_{off}, and k_{on}. The best way to produce such patterns is to simulate them using a numerical solution for eqs 5−10 supplemented with injection conditions (30). We found numerical solutions of these equations using COMSOL 4.3a commercial software. A program, based on such

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numerical solution, allows one to change basic parameters (K_d, k_{off}, k_{on}, v_A, v, A_{tot}, B_{tot}, and V_{inj}) and calculate simulated temporal propagation patterns for A + C. The latter can then be utilized to back-calculate K_d, k_{off}, and k_{on} by employing expressions 35 and 29. Since the true values of K_d, k_{off}, and k_{on} are known in this case, corresponding relative errors δ, δ_{off}, and δ_{on} (an absolute value of the ratio between the deviation from the true value and the true value) are easy to determine. Of course, the numerical solution for eqs 5−7 can also be used in the pattern-based approach to find K_d, k_{off}, and k_{on}; however, the latter requires a fitting procedure that is mathematically nontransparent and much more complicated than simple explicit expressions 35 and 29.

We simulated a total of 40 temporal propagation patterns of the mixture of A and C mimicking experimental signals. The patterns have the form shown in Figure 1a and correspond to various values of K_d, k_{off}, k_{on}, A_{tot}, and B_{tot} and to two different lengths, L = 15 and 30 cm, of the column. Excel file "Errors" (that can be downloaded from www.yorku.ca/skrylov/resources.html) shows: (i) values of A_{tot}, B_{tot}, K_d, k_{off}, and k_{on} used in COMSOL simulations as input data; (ii) areas of peaks A and C and tail A_{tail} obtained from the simulated signals; (iii) values of K_d calculated with eq 35; (iv) values of parameter λ calculated with eq 28; (v) values of k_{off} and k_{on} calculated with eqs 29; (vi) relative errors in determination of K_d, k_{off}, and k_{on}. These data give a step-by-step illustration of the computational process found in Figures 2−4. Values of t_{sep}/t_{eq} used in the simulations cover the interval of $0.001 < t_{sep}/t_{eq} < 0.1$ in Figures 2−4. In simulations, we also assumed that $g = g_A/g_C = 1$, W_0 = 0.125 cm, v = 0.060 cm/s, v_A = 0.033 cm/s, V_{inj} = 10 μL, and R = 0.23 cm, where R is the inner radius of the column. The corresponding value of t_{sep} is 4.6 s. Such values are typical for KSEC-based experiments. We used a real experimental value for coefficient $g_A/\pi R^2 = 88 \times 10^{15}$ cm/mol in relation 11.[20] For the reader's convenience, the Supporting Information also contains step-by-step instructions for how to use the developed method for practically finding K_d, k_{off}, and k_{on}.

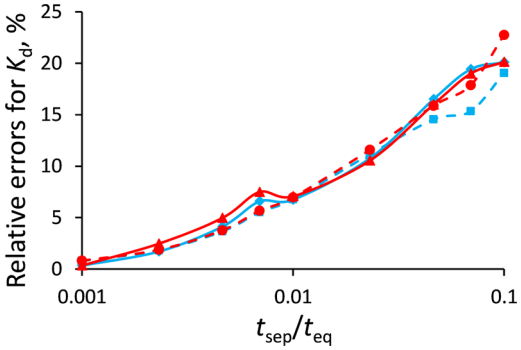

Figure 2. Influence of t_{sep}/t_{eq} on relative errors in determination of K_d for $B_0 = K_d$ (blue lines) and $B_0 = 2K_d$ (red lines). Results for the column length L = 30 and L = 15 cm are depicted by solid and dashed lines, respectively.

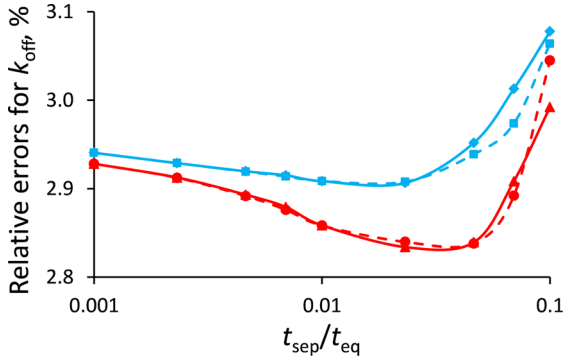

Figure 3. Influence of t_{sep}/t_{eq} on relative errors in determination of k_{off} for $B_0 = K_d$ (blue lines) and $B_0 = 2K_d$ (red lines). Results for column lengths L = 30 and L = 15 cm are depicted by solid and dashed lines, respectively.

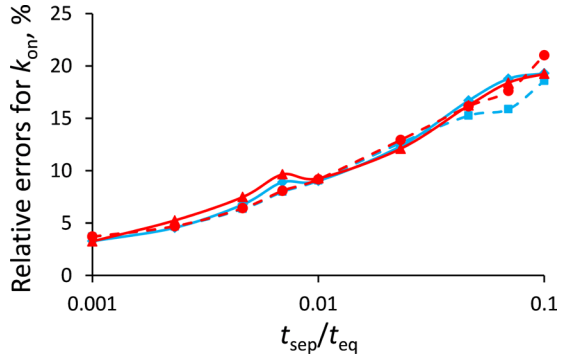

Figure 4. Influence of t_{sep}/t_{eq} on relative errors in determination of k_{on} for $B_0 = K_d$ (blue lines) and $B_0 = 2K_d$ (red lines). Results for column lengths L = 30 and L = 15 cm are depicted by solid and dashed lines, respectively.

Partially nonmonotonic or unsmooth behavior of curves in Figures 2−4 can be caused by errors in the measurement of areas S_C, S_A, and S_{tail} in Figure 1a. According to relations 28, 29, and 35, such errors can affect data analysis that is based on peak areas.

Applicability of the Slow-Equilibration Approximation to Determination of K_d, k_{off}, and k_{on}. The results of these 40 pseudoexperimental tests are presented in Figures 2−4. They show dependencies of the relative errors in determination of K_d, k_{off}, and k_{on} using the slow equilibration approximation. We used two different values of the ratio B_0/K_d = 1 and 2 (blue and red lines in Figures 2−4, respectively). To calculate K_d, we used expression 35 with values of s_A, s_C, and s_{tail} determined from the simulated signals. Corresponding errors in determination of K_d are shown by solid and dashed lines for L = 30 and 15 cm, respectively, in Figure 2. To calculate parameter λ determined by relation 28, we used simulations for both column lengths. Then, k_{off} and k_{on} values were calculated using relation 29 in which K_d values were determined either from simulations for L = 30 cm or from simulations for L = 15 cm. Corresponding errors in determination of k_{off} and k_{on} are depicted by solid and dashed lines for L = 30 and L = 15 cm, respectively in Figures 3 and 4. The upper limit of the t_{sep}/t_{eq} ratio in Figures 2−4 corresponds to simulated signals from the mixture of A and C, in which the peak C shown in Figure 1a would almost disappear for the longer length of L = 30 cm.

The data in Figures 2−4 show that the slow-equilibration approximation (based on relations 35 and 29) facilitates the determination of K_d, k_{off}, and k_{on} and ensures acceptable relative errors if $t_{sep}/t_{eq} < 0.1$. Errors for k_{off} remain around 3% in the entire range of t_{sep}/t_{eq} studied. Errors for K_d and k_{on} decrease from approximately 20% to 1% with a decrease in t_{sep}/t_{eq} from 0.1 to 0.001. Higher errors for k_{on} and K_d in comparison of that for k_{off} are likely explained by reassociation of B and A in the zone of C before A leaves this peak and by the fact that B is not

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constant in zone C. Higher errors in K_d should also result in higher errors in k_{on} due to the relation of $k_{on} = k_{off}/K_d$. The comparison of solid and broken lines in Figures 2−4 shows that relative errors are not significantly affected by the column length used in simulations. Systematic errors of 20% for K_d and k_{on} at the upper limit of t_{sep}/t_{eq} can be considered acceptable since a precision of experimental data is often worse than 20%. Actually, Figures 2−4 allow one to estimate errors of the developed method and recommend working at $t_{sep}/t_{eq} < 0.01$ to ensure small errors of ∼5%.

Basic Assumptions. Let us formulate again the basic assumption made in the derivation of the slow-equilibration approximation and the corresponding method for finding K_d, k_{off}, and k_{on}. We assumed that (i) the characteristic separation time is much smaller than the characteristic equilibration time (i.e., $t_{sep} \ll t_{eq}$; it should be also noted that solutions 21 are valid for both $t_{sep} \ll t_{eq}$ and $t_{sep} \sim t_{eq}$); (ii) the total amount of B in the injected equilibrium mixture is much larger than that of A ($b_{tot} \gg a_{tot}$); (iii) diffusion fluxes can be neglected at the boundaries of zone C since our estimates show that longitudinal Peclet number is very large due to the small diffusion coefficient of $D_P \sim 10^{-7}$ cm^2/s;[27] (iv) the mixture of A, B, and C is at equilibrium immediately before the injection; (v) $t_{inj} \ll t_{eq}$, where t_{inj} is the injection time and t_{eq} is the equilibration time; (vi) $t_{in} \sim R_{in}^2/D_{in} \ll t_{inj}$, where t_{in} is the characteristic time of diffusion relaxation between the concentration of small molecule outside the beads and inside their pores; (vii) average concentrations of small molecule outside the beads and inside their pores are approximately the same due to fast diffusion equilibration ($t_{in} \ll t_{sep}$). The slow-equilibration approximation allows one to avoid difficulty of solving inverse problems as in the pattern-based approach.[19,20] The slow-equilibration approximation will be a common case for tight binding with $K_d \sim 10^{-7}$−10^{-9} M ($k_{off} \sim 10^{-2}$−10^{-3} 1/s, $k_{on} \sim 10^5$−10^6 1/(Ms)) studied with concentrations of B being in the range of K_d (10^{-7}−10^{-9} M). As such, the simple solution will be applicable to a large number of practical cases.

CONCLUSIONS

In this work, we introduced a slow-equilibration approximation in KSEC that includes: (i) a new approximate analytical solution of mass-transfer equations for the nonequilibrium reaction 1 and (ii) a simple parameter-based method for finding rate constants k_{on} and k_{off} for this reaction. We considered a general case with two requirements: (i) two peaks can be identified in signals of reactants A and C (Figure 1a) and (ii) the protein was taken in an excess to the small molecule during the preparation of the equilibrium mixture ($b_{tot} \gg a_{tot}$). Then, we have an approximate analytical solution (21) for the amounts of components A and C in peak C. This solution allowed us to obtain expressions (29) for k_{off} and k_{on} in terms of parameter λ and the equilibrium constant K_d. Parameter λ can be found from relation 28 using measurements of areas of peak C for two different lengths L_1 and L_2 of the SEC column. The equilibrium constant K_d, in turn, can be found from a NECEEM-like procedure using expressions (35) and measurements of areas of peaks A and C and the area of A_{tail}.[27] We tested the accuracy of the slow equilibration approximation by applying it to 40 temporal propagation patterns simulated with the exact (numerical) solution of eqs 5−8. In the case of K_d and k_{on}, we found that the method's accuracy was better than 20% for $t_{sep}/t_{eq} < 0.1$. In the case of k_{off}, the method's accuracy was better than 3% for $t_{sep}/t_{eq} < 0.1$. Thus, the slow-equilibration approximation in KSEC provides a simple and effective way for studying rate constants of noncovalent interactions described by reaction 1.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b00415. The Excel file "Errors" used in preparation of Figures 2−4 with corresponding output data from COMSOL 4.3a commercial software and the Excel file "Examples" with corresponding examples can be downloaded from www.yorku.ca/skrylov/resources.html.

Procedure of obtaining rate constants from experimentally measured dependencies of signal on time using a slow-equilibration approximation in kinetic size exclusion chromatography (KSEC). (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Supporting Information

Slow-Equilibration Approximation in Kinetic Size Exclusion Chromatography

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ABSTRACT: Here we describe in detail a procedure of obtaining rate constants from experimentally measured dependencies of signal on time using a slow-equilibration approximation in Kinetic Size-Exclusion Chromatography (KSEC). We use Excel file "Examples" to illustrate the examples. The file can be downloaded from www.yorku.ca/skrylov/resources.html. In the main text, we also referred to Excel file "Errors" that can be also downloaded from the same site.

To obtain rate constants one should prepare a pre-equilibrated mixture of the small molecule (A) and the protein (B); the mixture also contains their complex (C). So, the total amounts of A and B in the injected plug (denoted by a_{tot} and b_{tot}, respectively) and the concentrations of A and B before equilibration (denoted by A_{tot} and B_{tot}, respectively) are assumed to be known. These quantities are related by equations:

$$A_{tot} = \frac{a_{tot}}{V_{inj}}, \quad B_{tot} = \frac{b_{tot}}{V_{inj}} \tag{S1}$$

Values of A_{tot} and B_{tot} (in nM) should be entered into cells B4 and D4 of the Excel file.

We also should know the injection flow rate v_{inj} and the injection time t_{inj}. Values of v_{inj} (in L/s) and t_{inj} (in seconds) should be entered into cells B1 and D1 of the Excel file. Using them we can find the injection volume V_{inj} (cell F1 in the Excel file)

$$V_{inj} = v_{inj} t_{inj} \tag{S2}$$

A signal at the column exit generated cumulatively by A and C should be measured. The signal must be of the kind shown in **Fig. 1(a)** that corresponds to $t_{sep} << t_{eq}$, otherwise the slow-equilibration approximation is not applicable to finding k_{on} and k_{off} in KSEC. Areas under peaks A, C, and A_{tail} in **Fig 1(a)**, denoted by s_A, s_C, and s_{tail}, respectively, in the main text, should be measured at two different column lengths, L_1 and L_2. If we have 9 experiments the corresponding areas should be entered into cells A8 – A16, B8 – B16, and C8 – C16 for L_2 and into cells G8 – G16, H8 – H16, and I8 – I16 for L_1 in the Excel file, where we use the following notations: s_C = C_area, s_{tail} = Tail_area, and s_A = A_area.

The K_d value can then be determined from a NECCEM-like procedure using expression (35) in the main text. Given relations (S1), we can determine K_d from the following equation:

$$K_d = \frac{B_{tot}\left(1 + \dfrac{s_A}{s_C + s_{tail}}\right) - A_{tot}}{1 + \dfrac{s_C + s_{tail}}{s_A}} \tag{S3}$$

For example, the found values of K_d (in nM) are shown in the cells D8 – D16 and J8 – J16 in the Excel file.

We then need to calculate the injected plug length W_0 using relations (10) in the main text:

$$W_0 = \frac{V_{inj}}{\pi \phi_{out} R^2}, \quad \phi_{out} = \frac{V_{free}}{\pi R^2 L} \tag{S4}$$

To find W_0, we should know the volume of the column accessible to the protein. This volume is close to the column free volume and is thus denoted by V_{free}. The value of V_{free} can be estimated from expression:

$$V_{free} = v_{inj} t_C \tag{S5}$$

Here t_C is the elution time of peak C that should be measured from the signal presented in **Fig. 1(a)**. Finally, we obtain the following expression for W_0:

$$W_0 = \frac{LV_{inj}}{V_{free}} = \frac{LV_{inj}}{v_{inj} t_C} \tag{S6}$$

where L is the column length that must be known and entered (in cm) into cells L3 and L4 of the Excel file. The corresponding value of W_0 (in cm) is calculated in cell H1 of the Excel file.

The next step includes the calculation of t_{sep} using expressions:

$$t_{sep} = \frac{W_0}{v_C - v_A}, \quad v_C = \frac{L}{t_C}, \quad v_A = \frac{L}{t_A} \tag{S7}$$

Here t_A is the elution time of the peak A that should be measured from the signal presented in **Fig. 1(a)**, v_C is the velocity of the protein and complex (v_C is denoted by v in the main text), v_A is the average velocity of the small molecule. Values of t_C and t_A (in seconds) should be entered into cells B2 and D2 of the Excel file. Values of v_C and v_A should be calculated using the second and third relations (S7) after the elution times of the peaks C and A are measured. Values of v_C and v_A are calculated in cells B3 and D3 of the Excel file. The value of t_{sep} is calculated in the cell H3 of the Excel file.

Using the slow-equilibration approximation in KSEC for determination of k_{off} and k_{on} requires measurements of signals for two different column lengths: L_1 and L_2 ($L_2 > L_1$). Corresponding areas of peak C in **Fig. 1(a)** are denoted by $s_C(L_1)$ and $s_C(L_2)$.

Then expression (28) should be used to determine an additional parameter λ:

$$\lambda = \frac{v_C}{L_2 - L_1} \ln \frac{s_C(L_1)}{s_C(L_2)} \tag{S8}$$

Here, v_C is the velocity of the protein and complex determined by the second relation (S7). For example, corresponding values of λ are calculated in cells M8 – M16 of the Excel file.

Finally, expressions (29) are used to find k_{off} and k_{on}:

$$k_{\text{off}} = \frac{3\lambda - t_{\text{sep}}\lambda^2}{3 - t_{\text{sep}}\lambda\left(1 + \dfrac{B_{\text{tot}}}{K_d}\right)}, \quad k_{\text{on}} = \frac{k_{\text{off}}}{K_{\text{d}}} \tag{S9}$$

Here, parameters λ, t_{sep}, and K_{d} were determined in the previous steps, B_{tot} is known from the procedure of plug preparation. For example, corresponding values of k_{off} are calculated in cells E8 – E16 and K8 – K16 of the Excel file. Corresponding values of k_{on} are shown in cells F8 – F16 and L8 – L16 of the Excel file.

At the end we should calculate t_{eq} using the second relation (13) in the main text:

$$t_{\text{eq}} = \frac{1}{k_{\text{on}} B_0 + k_{\text{off}}} \tag{S10}$$

where concentration B_0 in (S10) is determined by solving equations (12) in the main text:

$$A_0 B_0 = K_{\text{d}} C_0, \quad A_0 + C_0 = A_{\text{tot}}, \quad B_0 + C_0 = B_{\text{tot}} \tag{S11}$$

and k_{off} and k_{on} are already determined by (S9).

Then we should check if the following inequalities are valid:

$$t_{\text{sep}} << t_{\text{eq}}, \quad b_{\text{tot}} >> a_{\text{tot}} \tag{S12}$$

If conditions (S12) are satisfied then the slow-equilibration approximation in KSEC can be used to find rate constants k_{off} and k_{on}.