Accurate $K_d$ via Transient Incomplete Separation

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Current methods for finding the equilibrium dissociation constant, $K_d$, of protein-small molecule complexes have inherent sources of inaccuracy. We introduce “Accurate $K_d$ via Transient Incomplete Separation” (AKTIS), an approach that is free of known sources of inaccuracy. Conceptually, in AKTIS, a short plug of the pre-equilibrated protein-small molecule mixture is pressure-propagated in a capillary, causing transient incomplete separation of the complex from the unbound small molecule. A superposition of signals from these two components is measured near the capillary exit as a function of time, for different concentrations of the protein and a constant concentration of the small molecule. Finally, a classical binding isotherm – fraction of unbound small molecule vs protein concentration – is built and used to find accurate $K_d$ value. Here we prove AKTIS validity theoretically and by computer simulation, present a fluidic system satisfying AKTIS requirements, and demonstrate practical application of AKTIS to finding $K_d$ of protein-small molecule complexes.

Reversible binding of proteins (P) to small-molecule ligands (L) has an important role in regulation of cellular processes\(^4\). In addition, most therapeutic targets are proteins\(^2\), and drugs are developed to form stable PL complexes with them:

\[ P + L \rightleftharpoons PL \quad (1) \]

Complex stability is characterized by the equilibrium dissociation constant $K_d$, which is defined as:

\[ K_d = \frac{[L]_\text{eq} [P]_\text{eq}}{[PL]_\text{eq}} \quad (2) \]

where $[PL]_\text{eq}$, $[L]_\text{eq}$, and $[P]_\text{eq}$ are equilibrium concentrations of P, L, and PL, respectively. Finding accurate $K_d$ values of PL is pivotal for creating adequate models in systems biology and correctly ranking pharmaceutical hits in early stages of drug development\(^5\). All available methods for finding $K_d$ of PL have inherent (instrument-independent) sources of inaccuracy. For example, fluorescence spectroscopy (e.g. fluorescence anisotropy and fluorescence correlation spectroscopy) and thermophoresis require labeling L with a fluorophore\(^4\). Attaching a fluorescent label to a small molecule is virtually impossible without affecting its binding\(^3\) and, thus, the accuracy of $K_d$ measurements. Sensor-based techniques, e.g. surface plasmon resonance (SPR) and biolayer interferometry (BLI), require the immobilization of either L or P onto a sensor surface\(^5\). Attaching a molecule to a surface affects binding\(^7,8\) and, thus, accuracy of $K_d$ measurements. Isothermal titration calorimetry (ITC) does not require labeling or immobilization but has another source of inaccuracy: the heat of side reactions, such as binding of L to high-concentration impurities in P, binding of P to P, and solvation of protons released upon L’s binding to P\(^9\). An inherent source of inaccuracy in $K_d$ determined by direct mass-spectrometry (MS) is dissociation of PL during its transfer from the liquid or solid phase to the gas phase and during the ionization\(^10\). Accuracy of $K_d$ determination with Size-Exclusion Chromatography (SEC) is affected by inevitable adsorption of L, P, and/or PL onto the stationary phase of a SEC column\(^11\). Finally, finding $K_d$ via Taylor Dispersion Assay (TDA) in a capillary requires fast re-equilibration\(^12-14\) and, therefore, becomes inaccurate for stable PL, which re-equilibrate slowly.

As a result of inherent inaccuracies, $K_d$ values determined by different methods for the same PL may deviate by as much as orders of magnitude. Wätzig et al. stated in a recent review\(^15\): “Binding constants from different laboratories showed an average deviation of approximately 0.5 log units, which translates to a factor of about 3 by which the measurements differ. Surprisingly, this is just the average. The cut-off value not to keep two values in one data set was 2.5 log units which corresponds to a factor of more than 300 by which the measurements differ!” Such large inaccuracies in $K_d$ values can obviously lead to misinterpretation of experimental results, mistaken conclusions, and misconceptions. This problem motivated our search for an approach for finding $K_d$ of PL that is free of inherent sources of inaccuracy. Here, we propose “Accurate $K_d$ via Transient Incomplete Separation” (AKTIS), an approach for determination of $K_d$ of PL, for which we do not know inherent sources of inaccuracy. Conceptually, a short plug of the pre-equilibrated mixture of P and L is propagated in a capillary by pressure under laminar-flow conditions. Differential transverse diffusion of PL and L in the laminar flow leads to their very fast transient incomplete separation (TIS) in the longitudinal direction. A cumulative signal from protein-bound and protein-unbound L is integrated through the stream cross-section near the capillary exit for different initial concentrations of P and a constant initial concentration of L in the mixture. The signal-vs-time dependence is used to calculate a fraction of unbound L for every initial protein concentration. A classical binding isotherm, which is the dependence of the fraction of unbound L on the initial concentration of P, is built. Finally, a value of $K_d$ is found by fitting a theoretical binding isotherm into the experimental one with $K_d$ being a single fitting parameter.

In this proof-of-principle work, we present the theory underlying AKTIS. We conduct computer simulation of the processes involved in AKTIS to demonstrate that AKTIS can accurately recover $K_d$. We construct an experimental setup that can support AKTIS both with fluorescence and MS detection. Finally, we experimentally demonstrate determination of $K_d$ by AKTIS for two protein small-molecule complexes: bovine serum albumin (BSA)-fluorescein and alpha-1-acid glycoprotein (AGP)-alprenolol. Our results agree with the consensus data found in the literature. The largest variation in $K_d$ obtained for our measurements on different days with a single detection mode was two folds. Further, the $K_d$ value obtained with fluorescence and MS detection differed by a factor of two. We associate the latter difference with different efficiencies of signal integration through the flow stream cross-section in these two modes of detection. Most of future development of AKTIS should be of

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engineering nature and should aim at optimally coupling the AKTIS fluidic system with different detectors.

Results

General procedure for determination of $K_d$. In general, finding $K_d$ requires determination of a fraction $R$ of unbound $L$ in the equilibrium mixture (EM) of $L$ and $P$ with the initial concentrations $[L]_0$ and $[P]_0$:

$$R = \frac{[L]_0 - [L]}{[L]_0}$$

Finding $R$, in turn, requires a signal $S$ that must be a superposition of signals from $L$ and $PL$:

$$S = S_L \times R + S_{PL} \times (1 - R), S_L \neq S_{PL}$$

where $S_L$ and $S_{PL}$ are the signals of pure $L$ ($[L] = [L]_0$, $[PL] = 0$) and pure $PL$ ($[L] = 0$, $[PL] = [L]_0$), respectively; $S_L$ and $S_{PL}$ can be found in experiments with $[P]_0 = 0$ and $[P]_0 >> K_d$, respectively. The signal from pure $P$ must be negligible with respect to $S$ even for $[P]_0 >> K_d$.

From equation (4), $R$ can be experimentally determined via measuring three signals $S_L$, $S_{PL}$, and $S$:

$$R_{exp} = (S - S_{PL}) / (S_L - S_{PL})$$

$R$ can be expressed theoretically as a function of $K_d$, $[P]_0$, and $[L]_0$: $16$

$$R_{theo} = \frac{K_d + [P]_0 - [L]_0}{2[L]_0} + \sqrt{\left(\frac{K_d + [P]_0 - [L]_0}{2[L]_0}\right)^2 + \frac{K_d}{[L]_0}}$$

A standard way of finding $K_d$ is to determine $R_{exp}$ for a wide range of $[P]_0$ at a constant $[L]_0$, and to plot a binding isotherm: $R_{exp}$ vs $[P]_0$. Finally, this binding isotherm is fitted with equation (6) using $K_d$ as a fitting parameter $18$. 

TIS of $L$ from $PL$. We hypothesized that a method for determination of accurate $K_d$ could be built upon a deterministic phenomenon of TIS of solutes with different diffusion coefficients in a laminar pipe flow. TIS is a known phenomenon, which could be predicted as early as in 1910 upon the results of Griffiths’ experiments with pressure-driven propagation of a fluorescein plug in a narrow tube $17$. Further, TIS could be accurately modeled as early as in 1953 with Taylor’s convection-diffusion equations for a diffusive solute in a laminar pipe flow $18$. Finally, the deterministic phenomenon of TIS has been computer-simulated and experimentally studied in multiple works spanning over four decades $19-22$. These studies provided important conceptual pillars and technical details for our work.

TIS of species with largely different sizes, e.g. $L$ and $PL$ in our case, occurs always when a plug of their mixture is propagated within a laminar pipe flow in a capillary, which is much longer than the plug (Fig. 1a). Laminar pipe flow is established by a pressure difference between the capillary ends and has a characteristic parabolic profile of flow velocity $17$. The velocity is zero at the capillary walls and reaches its maximum in the capillary center. TIS of $L$ from $PL$ in the longitudinal direction is possible due to the difference in rates of transverse diffusion between small-size $L$ and large-size $PL$. PL that is near the capillary center will diffuse to the capillary wall slower than L and, thus, will be displaced longitudinally by the flow more than L. PL located near the capillary wall will diffuse to the capillary center slower than L and will be displaced longitudinally by the flow less than L. As a result, during a short transitional stage, a bulk of PL moves faster than a bulk of $L$, while a tail of PL moves slower than that of $L$. The separation is incomplete, i.e., the concentration profiles of $L$ and $PL$ do overlap, even during the transitional stage. The separation gradually dissipates, i.e., the concentration profiles of $L$ and $PL$ become symmetrical around the same symmetry axis, after the transitional stage.

Tracking TIS is practical with a flow system in which the inlet-to-detector distance $l$ is linked with the average flow velocity $v_{av}$, characteristic diffusion time of $L$, $\tau_L$, the volumetric TIS flow rate $Q$, and diffusion coefficient of $L$, $\mu_L$, as (Fig. 1b) $19$:

$$l = v_{av} \tau_L = Q / (\pi \mu_L)$$

where $v_{av}$ relates to $Q$ and the inner capillary radius $a$ as:

$$v_{av} = Q / (\pi a^2)$$

The characteristic time of diffusion from the capillary centre to the capillary wall is:

$$\tau = a^2 / \mu$$

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If TIS is tracked with a system depicted in Fig. 1b, concentration profiles of L and PL will be separated in the time domain as shown in Fig. 1c, left. Further, if a signal can be measured for each of L and PL at the capillary exit and is proportional to the average cross-sectional concentration of each of them, \( S_L \propto [L] \) and \( S_{PL} \propto [PL] \), then, the cumulative signal \( S \) satisfies equation (4) and can, thus, be used to determine \( R_{exp} \) with equation (5). This conclusion proves fundamental suitability of TIS for finding \( K_0 \) of PL.

Examples of the cumulative signal, measured at different values of \( R \), are illustrated in Fig. 1c, right. Traces showing the dependence of the cumulative signal on time, \( S(t) \), will be called “separagrams”. The choice of a time for measuring \( S \) should be governed by minimizing error in \( R_{exp} \). A simple rule is to maximize the value of \((K_0 - S_{0L})\) in the denominator of equation (5). Time corresponding to the second peak in the separagram, which is just under \( S_L \) (Fig. 1c, right), and according to equation (9) it is mere 0.1 s for L of a size of small-molecule drug (\( \mu_1 = 500 \text{ mm}^2/\text{s} \)) and for a capillary radius of \( a = 10 \mu m \) (such a capillary is commercially available).

We showed above that TIS is theoretically suitable for measuring \( R_{exp} \) and, thus, for finding \( K_0 \) of PL. Unlike both SPR and BLI, TIS does not require immobilization of P or PL. Unlike direct MS, TIS does not require preserving intact PL for detection. Unlike SEC, TIS does not require a stationary phase for separation. Unlike TDA, TIS is equally applicable to weak and stable complexes. Unlike both SEC and TDA, TIS is so fast that adsorption of P, PL, and L onto the wall will typically be negligible. Unlike ITC, TIS is immune to side reactions. Evidently, TIS is free of known sources of inaccuracy present in SPR, BLI, direct MS, SEC, TDA and ITC. Accordingly, we coin a name of “Accurate Kₐ via TIS” (AKTIS) for our approach which (i) utilizes TIS to measure \( R_{exp} \) as a function of \([P]_0\) resulting in a binding isotherm, and (ii) uses the fitting of this binding isotherm with equation (6) to find \( K_0 \).

In addition to being free of known sources of inaccuracy, AKTIS is not restricted to a single mode of detection; any detector that can measure a cumulative cross-sectional average signal from L and PL satisfying equation (4) is suitable provided that it has a sufficiently high signal readout speed and a concentration limit of quantitation below \( K_0 \) values of the studied complexes. A unique advantage of AKTIS is that TIS is based on processes, which are deterministic in nature. These processes can be accurately described by a system of equations for convection-diffusion and reversible binding reaction without any empirical coefficients. As a result, AKTIS separagrams can be accurately computer-simulated and performance of AKTIS can be fully studied in-silico before any experimental proof-of-principle is attempted.

AKTIS using a computer-simulated separagrams. TIS is based on three key processes: 1) longitudinal advection of L, P, and PL in a laminar pipe flow, 2) their transverse diffusion, and 3) reversible binding of L and P. Longitudinal diffusion can be neglected. We created a virtual AKTIS setup in COMSOL Multiphysics software and used this virtual setup to simulate separagrams under conditions similar to realistic ones in an envisioned proof-of-principle experiment (see Materials and Methods and Supplementary Note 1).

The virtual setup was used to simulate an experiment in which: 1) EM (equilibrium mixture of L and P) was prepared outside the capillary in a solvent with properties of water, 2) a short plug of EM (sample plug) was injected by pressure-driven flow with a small flow rate of 0.1Q into the capillary pre-filled with water, 3) a plug of water was injected into the capillary with a small flow rate of 0.1Q to slowly displace the sample plug from the capillary inlet, 4) water was continuously pumped into the capillary at a high flow rate of Q to induce TIS of L from PL and P, 5) concentrations of L, PL, and P (determined as integrals of quantities of L, PL, and P through a cylindrical finite-length capillary cross-section divided by the volume of this cylinder) were recorded at the capillary end as functions of time, and 6) a sum of concentrations of L and PL was used to construct a separagram. Here and further we assume that P and PL have identical diffusion coefficients due to only a small difference in their hydrodynamic sizes.

The simulation parameters and results are readily scalable to any feasible l and Q, as long as their ratio satisfies \( l/Q \approx 1/(a\pi_0) \) (obtained from equation (7))24-27. Thus, an experimental setup with, e.g. \( l = 50 \text{ cm} \), \( Q = 50 \mu\text{L/min} \), and sample-injection flow rate of \( 5 \mu\text{L/min} \) could be simulated with 50 times lower values (1 cm, 1 µL/min, and 0.1 µL/min) to reduce computational time by approximately a factor of 50 (from hours to minutes). The remaining parameters (diffusion coefficients, rate constants, concentrations, and capillary radius) were set at realistic values.

Representative separagrams simulated in the virtual experiment are shown in Fig. 2a. Signals required for determination of \( R_{exp} \) were obtained by averaging points within a time-window near \( t_0 \) (= 20 s). The middle of the window corresponded exactly to the maximum of the second peak (e.g. 16.63 s in Fig. 2a). The first point and last point of the window corresponded exactly to 0.96 and 1.04 of the maximum of the second peak (Materials and Methods), respectively (e.g. 15.97 s and 17.30 s in Fig. 2a). Further, the fraction \( R_{exp} \) of unbound L at a given intermediate value of \([P]_0\) was determined with a slightly modified equation (5):

\[
R_{exp} = \frac{S_{PL}}{S_L - S_{PL}} = \frac{S_{PL_{0}} - S_{PL_{0,0}}}{S_{PL_{0}} - S_{PL_{0,0}} - S_{PL_{0,0},0}}
\]

(10)

where \( S_{PL_{0}} \) is a signal at the intermediate value of \([P]_0\). Here and in the following, we approximate \( S_L \) with \( S_{PL_{0}} \) and \( S_{PL} \) with \( S_{PL_{0}} \). The main reason for such approximation is that strictly speaking \( S_L \) corresponds to \([PL] \approx [L] \), which cannot be achieved in a real experiment since it requires \([P] \to \infty \). Therefore \( S_L \) is approximated with \( S_{PL_{0}} \) and, symmetrically, \( S_{PL} \) is approximated with \( S_{PL_{0}} \).

The values of \( R_{exp} \) determined from the simulated separagrams in Fig. 2a with equation (10), were used to build a binding isotherm.
shown in Fig. 2b. Non-linear regression was used to fit the isotherm with \( R_{exp} \) represented by equation (6) with \( K_d \) being a single fitting parameter. The best fit is shown as a solid line in Fig. 2b. The \( K_d \) value that corresponded to the best fit differed from the one used in simulations by only 3%. The deviation from the ideal value was expected. Our virtual experiment included a number of non-idealities of a real experiment. For example, the initial plug after injection by finite pressure had finite length and a non-cylindrical shape. The limited number of points in the binding isotherm (like in a real experiment) also contributes to the observed deviation.

Thus, while static quenching will affect separagrams, its presence does not drastically affect the results. The resulting separagrams are shown in Fig. 2a and Supplementary Fig. 1. The values of \( \mu \) used in the simulations were calculated for a range of expected values (from 300 to 1500 \( \mu m \)) for organic molecules with MW < 1000 Da. In practice, the value of \( \mu \) is not known precisely. To study robustness of AKTIS to uncertainties in \( \mu \), we simulated separagrams with \( 1/Q \) ranging from 1/(\( 3\mu L \)) to 3/(\( 3\mu L \)), which is in the range of expected variations for \( \mu L = 500 \mu m^2/s \) chosen as the value for the first set of simulations (see Fig. 2c). The resulting separagrams are shown in Supplementary Fig. 1. They are qualitatively similar to those obtained for \( 1/Q = 1/(\mu L) \).

The values of \( R_{exp} \) were calculated for times corresponding to the maximum of the second peak (as in Fig. 2a) and \( K_d \) values were found not to deviate more than 5% from the value used in simulations, proving that AKTIS is robust with regards to the choice of \( 1/Q \), and suggesting that a single value of \( 1/Q \) can be used to study L greatly varying in size. Thus, our study of AKTIS applied to simulated separagrams showed that AKTIS is accurate and robust.

### Quenching and masking effects in signal detection

In our above-described in-silico study of AKTIS, we used a direct link between the signal and a cumulative concentration of L and PL: \( S = [L] + [PL] \). In reality, the signals from L and PL depend on the nature of both P and L as well as the mode of detection. P can influence the signal from L by binding L (we’ll call this influence “quenching”) and by being in the detector at the time of registration (we’ll call this influence “masking”).

If optical (e.g., fluorescence) detection is used, static quenching with a constant quenching coefficient is likely to be present. On the other hand, masking (i.e. dynamic quenching) in optical detection is unlikely. As a result, the signal in optical detection is multiplied with a constant quenching coefficient. Therefore, the signal will satisfy the requirement of signal superposition expressed by equation (4). Thus, while static quenching will affect separagrams, its presence will not affect the \( K_d \) value determined with the AKTIS procedure illustrated in Fig. 2.

If MS is used for detection of L, conditions should be created to dissociate PL during ionization to exclude the need for detecting the intact PL. The dissociation of PL will automatically exclude signal quenching. However, the presence of unbound P can affect ionization of unbound L and, in turn, signal from L, causing its masking (either increase or decrease in the signal). To “unmask” the signal, an operator \( O \), which describes a mathematical compensation procedure, should be applied to the raw MS signal \( S_{raw}(t) \):

\[
S(t) = O S_{raw}(t)
\]

Subsequently, this “unmasked” signal can be used for \( K_d \) determination using the standard AKTIS protocol.

A simple masking-compensation procedure can be built upon two facts. First, P and PL have similar separagrams; these separagrams can be robustly and accurately computer-simulated. Second, the concentration (and amount) of L is constant in experiments with varying [P]; thus, the areas under the separagrams should be constant. Based on these two facts, we suggest a compensation procedure with two operations: 1) multiplication (operator \( O_1 \)) of the measured separagrams by the simulated profile of P and 2) subsequent normalization (operator \( O_2 \)) of the separagrams to make the areas (integrals) under \( K_d \) equal to the area (integral) under the experimental separagram of L corresponding to the smallest amounts of protein \( \{[P] \ll [K_d] \} \):

\[
\tilde{O} = O_1 O_2 \tilde{O}_{M}, \quad \tilde{O}_M = \tilde{S}_p(t), \quad \tilde{O}_N = \frac{\int \tilde{S}_p(t) S_{PPL > K_d}(t) dt}{\int \tilde{S}_p(t) S_{PPL}(t) dt}
\]

where \( \tilde{S}_p \) is the dimensionless simulated separagram of pure P. \( \tilde{O}_N \) can be greater or smaller than unity, i.e. the presence of P can either suppress or enhance ionization of L. Combining equation (11) with equation (12) provides an instruction on how to process the raw signal in order to get the unmasked signal for \( K_d \) determination:

\[
S(t) = \frac{\int \tilde{S}_p(t) S_{PPL < K_d}(t) dt}{\int \tilde{S}_p(t) S_{PPL}(t) dt} \tilde{S}_p(t) S_{PPL}(t)
\]

This unmasked signal can be used in equation (10) to find \( R_{exp} \) and determine \( K_d \).

### Fluidic system for AKTIS

Our in-silico studies proved that AKTIS can be used for finding accurate \( K_d \) of reversible protein-small molecule binding provided that an experimental setup can be built to conduct experiments similar to the simulated ones. In an experimental implementation of AKTIS we attempted to mimic in-silico studies in which plugs of identical volumes were injected by a constant low flow rate and TIS was carried out by a constant high flow rate. Our fluidic system is shown schematically in Fig. 3. In this system, the constancy of flow rates for sample injection and TIS is achieved by using two continuously running pumps under constant backpressures: the low-pressure pump (LPP) for sample transfer from the injection loop into the separation capillary and high-pressure pump (HPP) for TIS. Constant back-pressures are achieved, in turn, by using a mock fluidic circuit: a mock injection loop and a mock capillary of dimensions identical to those of the main injection loop and the separation capillary.

The operation of the AKTIS fluidic system involved three major stages with a total duration of 86 s. Transitions between these stages were carried out by switching fluidic connections with control valves; the switch time did not exceed 0.1 s. Stage 1 was the loading of the injection loop with the sample during 12 s (Fig. 3a). The sample was slowly pumped through the loop by the sample pump. The volume of the pumped sample was equal to three volumes of the loop; the excess of the sample was collected into the waste reservoir. During this stage, the HPP was pumping the buffer through the separation capillary and cleaning the capillary, and the LPP was pumping the sample through the mock fluidic circuit. Stage 2 was sample-plug transfer into the separation capillary during 24 s (Fig. 3b). The LPP was pumping the buffer into the injection loop filled with the sample and slowly transferring the sample from the injection loop into the separation capillary. It is known that a velocity profile of laminar flow is distorted at the capillary entrance open to a reservoir of a different diameter; therefore, the sample plug was injected into the capillary so that its back was at a distance from the capillary inlet much greater than the capillary diameter (3.2 vs 0.02 cm in our case). During this stage the sample pump was idle.
Supplementary Figs. 5,6 and the HPP was pumping the buffer through the mock capillary. Stage 3 was sample-plug propagation by HPP and, thus, TIS of PL from L registered with a detector; the sample pump is idle (not shown), and LPP is pumping the buffer through the separation capillary. The beginning of stage 3 was used as a starting point for the corresponding separagram. More details on the AKTIS fluidic system can be found in Materials and Methods and Supplementary Fig. 2.

### AKTIS with fluorescence detection.

The first set of AKTIS experiments was performed with BSA (P) and fluorescein (L) using a fluorescence detector. The obtained separagrams had typical TIS shapes with two peaks. The results revealed high repeatability (Supplementary Fig. 3). Equation (10) was applied to experimental data from Fig. 4a, and a binding isotherm was constructed (Fig. 4b). Non-linear regression of the isotherm with equation (6) gave $K_d = 28 \pm 6 \text{ mM}$. When reproduced on other days, the results varied between $12 \pm 3 \text{ mM}$ and $26 \pm 3 \text{ mM}$ (Supplementary Fig. 4).

### AKTIS with MS detection.

We coupled our AKTIS setup with an MS detector. To facilitate dissociation of PL during ionization, we utilized an Atmospheric Pressure Chemical Ionization source (see Materials and Methods). AKTIS-MS was used to determine $K_d$ for two protein-ligand complexes: BSA-fluorescein and AGP-alprenolol (Fig. 5). The AKTIS-MS measurements revealed opposite masking effects for these two binding pairs: MS signal from fluorescein decreased with increasing [BSA]$_0$ (Fig. 5a, left), while MS signal from alprenolol increased with increasing [AGP]$_0$ (Fig. 5b, left). Separagrams were highly repeatable (Supplementary Figs. 5,6). We applied our two-step signal-unmasking procedure to both sets of separagrams and obtained unmasked separagrams with characteristic TIS features (Figs. 5a,b, middle). Equation (10) was then used to build binding isotherms (Figs. 5a,b, right). Finally, equation (6) was used to fit the isotherms and find $K_d = 31 \pm 4 \text{ mM}$ for the BSA-fluorescein complex and $K_d = 1.4 \pm 0.2 \text{ mM}$ for the AGP-alprenolol complex. The largest deviation from these values obtained on different days did not exceed two folds: $K_d = 44 \pm 4 \text{ mM}$ for BSA-fluorescein and $K_d = 2.7 \pm 0.6 \text{ mM}$ for AGP-alprenolol (Supplementary Figs. 7,8). The $K_d$ value for BSA-fluorescein obtained with AKTIS-MS is approximately two times higher than the value obtained by AKTIS with fluorescence detection. Our $K_d$ values agree with the literature values for both BSA-fluorescein (10-70 µM) and AGP-alprenolol (2-35 µM) complexes.

### Discussion

In this proof-of-principle work, we introduce AKTIS as a generic approach for measuring accurate $K_d$ of protein-small molecule complexes. While relying on separation, AKTIS does not require any form of stationary phase and does not impose any requirements on the protein and the small molecule except for the requirement of their different sizes. AKTIS does not require immobilization of the protein or small molecule. TIS can be achieved even in a sub-second time scale, making AKTIS applicable to very unstable protein-ligand complexes. On the other hand, AKTIS is perfectly applicable to stable complexes. The short time of TIS should prevent inaccuracies associated with adsorption of the protein-ligand complex or the ligand onto the capillary wall. In the case of severe adsorption that influences the results, the capillary inner wall can be passivated with any kind of chemistry; TIS should not be affected by such capillary
Fig. 5. Determination of \( K_0 \) by AKTIS with MS detection. a, Determination of \( K_0 \) for the BSA-fluorescein complex; \([\text{fluorescein}]_0 = 0.2 \, \mu\text{M} \) and \([\text{BSA}]_0 \) ranges from 0 to 0.5 mM. MS signal for fluorescein was measured at \( m/z = 287 \). b, Determination of \( K_0 \) for the AGP-alprenolol complex; \([\text{alprenolol}]_0 = 0.5 \, \mu\text{M} \) and \([\text{AGP}]_0 \) ranges from 0 to 0.2 mM. MS signal for alprenolol was measured at \( m/z = 250 \). Left: representative raw separagrams. Middle: separagrams after application of the signal-unmasking procedure; the vertical dashed lines show the time window at which an averaged signal was taken to calculate \( R_{\exp} \) with equation (10). Right: binding isotherms \( R_{\exp} \) vs \([P]\), open circles and their best fits (solid lines) obtained with equation (6). Conditions for AKTIS were similar to those described in the legend to Fig. 4 except for \( l = 100 \, \text{cm} \) and \( Q = 100 \, \mu\text{L}/\text{min} \) (\( l/Q \) was the same). The uncertainty of \( R \) was obtained by error propagation (Supplementary Note 3).

Materials and Methods

General. All chemicals and buffer components were purchased from Sigma-Aldrich (Oakville, ON, Canada). Fused-silica capillaries were purchased from Molex (Polymeric Technologies, Phoenix, AZ, USA). All measurements were carried out at room temperature (22 ± 2 °C). A single buffer, 30 mM ammonium acetate pH 7.5, was utilized to prepare all solutions. When we refer to the buffer, we mean 30 mM ammonium acetate pH 7.5.

Simulation of separagrams. To simulate separagrams we used COMSOL Multiphysics software, version 5.3, with the “Transport of Diluted Species” module, which incorporates mass transfer equations (the model is available in the Supplementary files). The respective system of differential equations with initial and boundary conditions are shown in Supplementary Note 1. The laminar flow can be obtained if the Reynolds number, \( Re = 2Q/(\nu L) \), is less than a thousand, where \( Q \) is the volumetric flow rate, \( \nu \) is the kinematic viscosity of a liquid, and \( L \) is the capillary internal radius. As a result, water (\( \nu \approx 10^{-6} \, \text{m}^2/\text{s} \)) will have laminar flow in narrow capillaries (\( a \approx 100 \, \mu\text{m} \)) as long as \( Q \approx 1 \, \text{mL}/\text{min} \). The simulation parameters were chosen to ensure that the flow is laminar.

The simulation parameters were also chosen in order to have the detection time of the peak of L corresponding to the characteristic diffusion time of L as discussed in the main text (see equation (9)). This condition resulted in the following ratio: \( l/Q \approx 1 \) (\( l/Q \)). Considering L with \( \mu_L = 500 \, \mu\text{g}\text{mL}^{-1} \), and a capillary with an internal radius \( a = 100 \, \mu\text{m} \), the detection time would be 20 s, which is a reasonable value for the prototype experimental setup. Computational time depends on the dimensions of the simulated capillary. Thus, to reduce this time, the inlet-to-detector distance \( l \) can be scaled down while the ratio \( l/Q \) is kept constant. \( l = 1 \, \text{cm} \) and detection window of 0.1 mm were chosen which resulted in the flow rate of \( Q \approx 0.9 \, \mu\text{L}/\text{min} \) and for simplicity \( Q = 1 \, \mu\text{L}/\text{min} \) was used. Sample plug injection flow rate should be smaller than TIS flow rate. Thus, sample-plug injection was done with a flow rate of 0.1 \( \mu\text{L}/\text{min} \) during 12 s resulting in a near-rectangular injection plug

Fig. 4

Supplementary Note 1

Supplementary Note 3
of approximately 0.6 mm in length. Subsequently, a water plug was injected into the capillary with 0.1 µL/min during 12 s to slowly displace the sample-plug from the capillary inlet (plug end distance from the capillary inlet was approximately 0.6 mm). The rest of simulation parameters were as follows: \( k_{a1} = 10^{-5} \text{ s}^{-1} \), \( k_{a2} = 10^4 \text{ M}^{-1} \text{s}^{-1} \), \( K_2 = k_{a2}/k_{a1} = 1 \text{ µm} \), \( \nu_{p} = \nu_{p0} = 50 \text{ µm}^2/\text{s} \), \( [L_p] = 0.5 \text{ µM} \), temperature = 293.15 K (used by COMSOL to define physical parameters of water, e.g. viscosity and density). \( [P_0] \) was varied from 1 nM to 1 mM using 11 different concentrations plus a run at \( [P_0] = 0 \). Further, to study robustness of AKTIS to uncertainties in \( \mu_L = 500 \text{ µm}^2/\text{s} \) we simulated separagrams with 1/1 \( Q \), 1/3 \( Q \), 1/2 \( Q \), 2 \( Q \), and 3 \( Q \) for a setup with MS detection. Two mock capillaries were used to allow the continuous operation of LPP and HPP and ensure that they ran under constant back-pressures.

The pumps operated at the following flow rates: the sample pump at 15 µL/min, LPP at 5 µL/min, HPP for fluorescence detection at \( Q = 50 \text{ µL/min} \) and HPP for MS detection at \( Q = 100 \text{ µL/min} \) (to keep \( 1/Q = \text{const} = 1 \text{ cm min} \text{ µL}^{-1} \)). An 86-s-long measurement cycle included 3 stages. Switching between the stages was done by changing positions of the valves during less than 0.1 s. For the very first run and cycle, the sample was pre-filled into the capillary connecting the sample pump and Valve 1 (using its position II) before starting the cycle; in subsequent runs and cycles, this pre-filling was done during the third stage.

The first stage was 12-s-long sample loading into the 1-µL injection loop (Supplementary Fig. 2a). Valves 1 and 2 were in position I. The sample pump moved sample volume equal to \( 3 

\times V_{loop} \) through the injection loop to insure its complete filling. LPP was pumping the buffer through the mock loop and mock capillary. HPP was pumping the buffer through the separation capillary.

The second stage was 24-s-long sample transfer from the injection loop into the separation capillary (Supplementary Fig. 2b). Both valves were in position II. LPP moved the buffer \( \nu_L \) of the sample-containing injection loop. The sample was subsequently transferred into the separation capillary. At the end of this stage a sample plug of approximately 3.2 cm in length was 3.2 cm away from the inlet of the separation capillary. HPP was pumping the buffer through the mock capillary. The sample pump was idle.

The third stage was 50-s-long sample propagation through the separation capillary (Supplementary Fig. 2c). Valve 2 was in position I, while Valve 1 stayed in position II. The sample pump was used for pre-filling. HPP pumped the buffer into the separation capillary for TIS of PL from L. LPP was pumping the buffer through the injection loop. In total the injection loop was rinsed with \( 6 \times V_{loop} \) \( (2 \times V_{loop} \) during the second stage and \( 4 \times V_{loop} \) during the third stage).

Fluorescence detector. A diode-pumped solid state laser (AixiZ, Houston, TX, USA) was a light source for excitation of fluorescence. The laser beam had a diameter of about 2 mm and a power of 60 mW at 473 nm. Two subsequent neutral filters of 0.4 and 1.0 optical density (NE04B and NE10B, Thorlabs, Newton, NJ, USA) were used to attenuate laser power to \( \approx 2 \text{ mW} \). Fluorescence emission was collected by an MPlan 60x objective lens (NA = 0.7 at 90°) with an additional optical bandpass filter of 525 ± 25 nm (Semrock, Rochester, NY, USA). A photocathode of the photomultiplier tube R1477 (Hamamatsu Photonics, Hamamatsu, Japan) was biased at \( \approx 400 \text{ V} \). A 20 Hz low-pass electronic filter was introduced between the photomultiplier tube and the analog-to-digital signal converter PCI-6035E (National Instruments, Austin, TX, USA), to reduce electronic noise. Fluorescence data collection was controlled with the same custom LabVIEW software that was used to control the valves in the fluidic setup.

Fluorescence-based determination of \( K_4 \) for the fluorescein-BSA complex. Concentration of fluorescein was 0.2 µM. Concentration of BSA ranged from 0.1 µM to 1 mM. Fluorescein-BSA mixtures were vortexed and incubated at room temperature for \( \approx 2 \text{ h} \) to establish equilibrium in the reversible binding reaction. After the incubation, the EMs were subjected to AKTIS as described in the “AKTIS fluidic setup” section. To obtain \( R_{eq} \) for each BSA
concentration the experiment was repeated at least 5 times. The value of $K_d$ was obtained by fitting $R_{\text{exp}}$ vs $[P]_0$ with $R_{\text{noneq}}$ vs $[P]_0$ as described in the “Data acquisition and treatment” section.

**MS detector.** The output of the separation capillary was inserted into the MS ionization source (Turbomass ion source with APCI probe, AB Sciex, Vaughan, ON, Canada). MS detection was done with a QTRAP 6500+ instrument (AB Sciex, Vaughan, ON, Canada). The optimal acceleration and focusing conditions were achieved by using a 60-V declustering potential at 525 °C and 90-psi gas pressure. The MS analyses were performed in positive mode, and the analyzed m/z signals were processed using Analyst Q3 2.0 software. MS data collection was controlled with the same custom LabVIEW software and PCI-6035E board that were used to control the valves in the fluidic setup.

**Determination of $K_d$ for fluorescein-BSA and alprenolol-AGP complexes by AKTIS with MS detection.** The experiments were performed to measure $K_d$ of complexes between BSA (0.1–500 μM) and fluorescein (0.2 μM) as well as between AGP (0.1–500 μM) and alprenolol (0.5 μM). BSA-fluorescein and AGP- alprenolol mixtures were incubated at room temperature for $\approx 2$ h to establish equilibrium in the reversible binding reaction. After incubation, the EMs were subjected to AKTIS as described in the “AKTIS fluidic setup” section. Measurements of $R_{\text{exp}}$ for each protein concentration were done in triplicates. $K_d$ was obtained by fitting $R_{\text{exp}}$ vs $[P]_0$ with $R_{\text{noneq}}$ vs $[P]_0$ as described in the “Data acquisition and treatment” section.

**Data acquisition and treatment.** The experimental data acquisition was triggered at the beginning of the sample-transfer stage (second stage); this mimicked the way of treatment of simulated separagrams. The acquired or simulated data were evaluated using Excel and OriginPro (files are available in the Supplementary). For each experimental signal of the MS data the background taken at the beginning of the recorded separagram ($t \leq 5$ s) was subtracted; the background of the fluorescence data was already within the limits of signal noise and, hence, was not subtracted. Equation (10) was used to calculate $R_{\text{exp}}$. Signals required for determination of $R_{\text{exp}}$ were obtained by averaging points within a time-window near $t_0 \approx 20$ s. The middle of the window corresponded exactly to the maximum of the second peak ($e.g.$, 16.63 s in Fig. 2a) taken from the separagram of the smallest $[P]_0 \neq 0$. The first point and the last point of the window corresponded exactly to 0.96 and 1.04 of the position of this maximum of the second peak, respectively ($e.g.$, 15.97 s and 17.30 s in Fig. 2a). This window width was chosen to increase the signal-to-noise ratio while covering only the tip of the peak; for our data, the window width ranged between 1.2 and 1.5 s. The averaged signal at each concentration of sample was measured/simulated $n \geq 3$ times. The standard deviation ($\sigma$) for each $R_{\text{exp}}$ was obtained by simple error propagation based on equation (10) (Supplementary Notes 2 and 3). $K_d$ was obtained by fitting the dependence of $R_{\text{exp}}$ on $[P]_0$ with equation (6) (see main text). A weighted non-linear fitting was performed using the Levenberg-Marquardt algorithm; each point had a weight of $\sigma^{-2}$.

**Data and model availability.** The COMSOL model as well as the raw data and evaluated data for separagrams and binding isotherms in Figs. 2, 4, and 5 and Supplementary Figs. 1, 3-8 are provided as Supplementary files to the paper.

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**Author contributions**

N.S. performed the experiments, analyzed the data, developed software, and wrote the manuscript. J.L.R. performed the experiments, analyzed the data, developed software, and wrote the manuscript. J.C., Y.L.B. and J.B. helped with MS experimental design and MS experiments. V.A.G. and A.S.S. helped with fluorescence experiments, developed software, and wrote the manuscript. S.K. analyzed data, designed figures, helped with the mathematical formulation, and wrote the manuscript. S.N.K. conceptualized, conceived, and guided the study, designed experiments, suggested design of the AKTIS instrument, proposed the masking-compensation procedure, interpreted the data and wrote the manuscript. All authors reviewed and approved the manuscript.

**Competing interests**

The authors declare no competing financial interests.