SUPPLEMENTAL INFORMATION

Title Page

A rebinding-assay for measuring extreme kinetics using label-free biosensors

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Supplementary methods

Principle of rebinding-assay. Smoluchowski[S1] introduced a statistical description of kinetics in a microscopic framework that described diffusion-controlled association of bimolecular complexes. Later Bergs[s2] showed that when the association rate is in the same order, or higher, than the diffusion limited escape rate, then multiple unbinding-rebinding cycles can occur before the ligand fully escapes. It was also shown that self-rebinding caused the observed ligand dissociation rate constant to be slowed relative to the true chemical dissociation rate and competitive inhibition prevented self-rebinding. In addition to self-rebinding, rebinding can manifest in complex regimes where multiple receptors are available and is dependent on the size of the binding site relative to the distance between them, the volume of the mesoscopic region and the kinetics of the interaction[s3, s4].

Fig. 1. Diffusion of soluble species within the hydrogel was assumed to be 10-fold slower than in the bulk liquid. Other simulation parameters were as follows: **A** was injected at 100 µM over a serial 5-fold range in k_a from 1×10^5 $M^{-1}s^{-1}$ to 7.81×10^9 $M^{-1}s^{-1}$, $k_a' = 1 \times 10^6$ $M^{-1}s^{-1}$, $h = 200$ nM and $\mathbf{P} = 10$ mM.

Fig. 2: Da decreases on approach to saturation as observed by increasing curvature and the values shown represent the upper limit at the onset of binding where R = 0. The mass transport limited binding curves showing both association and dissociation phases were stimulated using Biaevaluation 4.1.1 (Cytiva life sciences) by selecting a two-compartment mechanistic interaction model and numerical integration of the associated coupled set of ordinary differential equations (ODE) given as;

$$\mathrm{B(solution)} = \mathrm{Conc}$$
$$\mathbf{B}[0] = 0$$
$$d\mathbf{B}/dt = k_t*(\mathrm{Conc}\text{-}\mathbf{B}) - (k_a*\mathbf{B}*\mathbf{P} - k_d*\mathbf{BP})$$
$$\mathbf{P}[0] = R_{max}$$
$$d\mathbf{P}/dt = -(k_a*\mathbf{B}*\mathbf{P} - k_d*\mathbf{BP})$$
$$\mathbf{BP}[0] = 0$$
$$d\mathbf{BP}/dt = (k_a*\mathbf{B}*\mathbf{P} - k_d*\mathbf{BP}).$$

The inhibition curves obtained for injection of inhibitor during the dissociation phase shown in the inset were modeled using the full computational model described below.

Fig. 3. Numerical simulation parameters: At $t = 0$, **B** is pre-bound to the hydrogel to a concentration equivalent to $R_0 = 300$ RU $= 0.1R_{max}$. At $t = 10$s, 0.5 mM **A** is injected inhibiting rebinding of **B** thereby accelerating escape of **B**. The inhibition curves were simulated over serial 1.5-fold increasing k_a and included a blank inhibition curve, where $\mathbf{A} = 0$. Other simulation parameters were as follows: $k_d = 0.001$ s^{-1}, $k_d' = 0.05$ s^{-1}, $h = 10$ µm, $\nu_c = 0.15$ m/s. Relative error $=$ 1-(true_k_a.**A**/k_a.**A**).

Fig. 5. Unless otherwise stated all other aspect of the simulation were performed as described for the virtual instrument parameter settings described below.

(a) All 48 combinations of the following parameters were simulated. Range in k_a was 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 ($M^{-1}s^{-1}$), range in k_d was 0.1,1,10 (s^{-1}) and $k_{inact} = 1$ s^{-1}.

(b) Range in k_d was covered as serial 1.5-fold increasing values over the range from 10^{-1} to 10^5. Single value of $k_a = 1 \times 10^6$ (M^{-1} s^{-1}).

(c-d) k_a and k_d were fit globally with all other parameters held constant for **A** = 1 mM and at flow velocities 0.1 m/s, 0.01 m/s, 0.001 m/s. Rather than determining separate values of k_t at each flow velocity, we employed a single value of t_c over all flow velocities, where $k_t = t_c*u^{1/3}$, and t_c is the flow rate-independent

mas transport coefficient. Optionally, such curves sets allow k_t, k_a and k_d to be estimated as globally constrained values.

(e) Single flow rate = $\nu_c = 0.1\ \mathrm{m/s}$, $A = 0$. Four curves representing four R_0/R_{max} values of 0.125, 0.250, 0.500, 1.000. Curves were superimposed by normalizing each curve to a unit curves allowing divergence to be visualized.

(f) Performed as described in (c-d) but over a range in R_0/R_{max} values of 0.0156, 0.0312, 0.0625, 0.125, 0.250, 0.500, 1.000. Fitting of equation (4) was also performed with k_d held constant at its true value. k_a was held constant at true value to reveal systematic error in k_d estimation.

Fig. 6. The simulation parameters for each assay format were matched in terms of R_0, R_{max}, kinetic range tested and a baseline noise of 0.06 RU was added to all response curves. Kinetic parameter values were chosen pseudo randomly over many orders in both k_a and k_d to producing large sets of simulated response curves. The range in simulated k_a values were defined by the limit $4 \leq \mathrm{Log}(k_a) \leq 9$ as this range represents all diffusion limited association reactions with an upper limited bounded by the maximal diffusion rate in aqueous phase and a lower limit bounded by the onset of conformationally gated binding. The k_a was varied over 10-orders spanning the upper and lower limits of practical importance in drug discovery where limit = $-6 \leq \mathrm{Log}(k_d) \leq 4$. Competitive kinetic inhibition curves were simulated over 6 serial 10-fold dilutions of inhibitor from 1 mM in order to compensate for intrinsically lower measuring range while rebinding was performed over just three serial 10-fold dilutions of inhibitor from 1 mM, where each was repeated at two injection flow rates giving two k_t values (i.e. $1000\ s^{-1}$ and $350\ s^{-1}$) per concentration. Mote Carlo simulations were then generated, with random baseline noise added to equivalent experimentally measured levels, where each simulated curve set was then back-fit to its parent model in order to test the accuracy of parameter return, measuring range and detection sensitivity. Both models were applied using same constraints when fitting. k_a and k_d were fit globally and k_t was fixed. The number of simulations included in each plot varies because simulation range exceeds the ranges plotted and was required by the Monte Carlo routine available. All parameters were fixed other than k_a and k_d in both simulations. In a drug discovery setting, the minimal resolvable response change defines the limit of detection for weak binding compounds and extending this range is of considerable value. Only the highest concentration of 1 mM was required for sensitivity analysis where the response was measured over an average of 4 points at the end of each 5 min injection and a cut-off response of 0.5 RU was selected. The resulting maximal responses were then plotted on an affinity space plot defined by the true k_a/k_d combinations of each simulated curve set and the results for competitive kinetics are shown in Fig. 6a and those for rebinding kinetics in Fig. 6b.

Virtual Instrument

Parameter settings. $R_0 \approx 0.1.R_{max}$ (RU) , $\mathbf{A} = 1$ (mM), $k_a = 1 \times 10^6$ ($M^{-1}s^{-1}$), $k_d = 0.001$ (s^{-1}), $k_a' = 1 \times 10^7$ ($M^{-1}s^{-1}$), $k_d' = 0.05$ (s^{-1}), $k_{inact} = 1$ (s^{-1}), $M_{r\_B} = 30$ (kDa), $M_{r\_A} = 200$ (kDa), $\mathbf{P} = 1$ mM (equivalent to $R_{max} = 3{,}000$ RU when fully saturated by **B**).

Instrument settings. $v_c = 0.1$ m/s, flow cell height (h) $= 20$ μM, flow cell length (l) $= 0.5$ mm, sensing region = hydrogel domain = 0.2 mm x 200 nm. Detection reports the average change in concentration of a given species within the hydrogel domain. The three-dimensional hydrogel is well approximated using a two-dimensional geometry because contributions near the flow cell walls can be neglected since the flow cell is thin relative to its width and parameter units are nevertheless provided in familiar three-dimensional form. The data collection rate was 1 Hz and baseline noise equivalent to 0.003 RU (root mean square) was added to response curves to mimic actual instrument performance.

Hydrogel modeling. A 200 nm thick hydrogel was modeled as a volume containing a homogenous density of hydrogel grafted to the sensing surface that decreases rapidly at the hydrogel-liquid interface according to the hydrogel density function density = $1\text{-Exp}(-100*(1\text{-z}))$, where z is the dimensionless relative height of the hydrogel. The concentration of **P** is assumed to be scaled by the hydrogel density and the diffusion coefficient of all species tethered to the hydrogel (i.e. **P**, **BP**) is assumed to be zero. Diffusion of all species inside hydrogel is assumed to be 2-fold lower due to a 2-fold increase in viscosity within the hydrogel relative to the bulk liquid and soluble species are subject to molecular weight-dependent partitioning. Therefore, parameters related to mass transport of soluble species inside the hydrogel are defined as follows. Diffusion coefficient of $\mathbf{A} = D_A = 5 \times 10^{-10}$ (m^2/s), diffusion coefficient of $\mathbf{B} = D_B = D_A(M_{r\_B} / M_{r\_A})^{1/3}$, diffusion coefficient of **A** inside hydrogel $= D_{gel} = 2.D_A.K_{part}$, where the hydrogel partition coefficient for $\mathbf{A} = K_{part} = \text{Exp}(-10^{-3} * M_{r\_A}{}^{2/3})$, diffusion coefficient of all soluble species containing **B** (i.e. **B**, **AB**, **AB***) inside hydrogel $= D_{gel} = 2.D_B.K_{part}$, where the hydrogel partition coefficient for $\mathbf{B} = K_{part} = \text{Exp}(-10^{-3} * M_{r\_B}{}^{2/3})$. The initial conditions for the simulation include addition of tethered **P** some fraction of which is in the form of affinity complex **BP** before the onset of the inhibitor injection. The inhibitor injection was simulated as a sample pulse entering from one end of the rectangular flow cell and exiting at the opposite end, where the tethered hydrogel film is located at one of the flow cell walls and is parallel to the direction of flow.

Finite element analysis. Coupled ODEs were solved numerically coupled to the master equations, which are partial differential equations (PDEs) governing flow, advection, diffusion, and reaction for a 20 μM thick flow cell housing a sensing region containing a hydrogel film functionalized with P. The entire geometry was discretized in space and solved over incremental time periods to generate surrogate experimental data. Comsol multiphysics 5.1 (COMSOL AB, Tegnérgatan 23, SE-111 40, Stockholm, Sweden) was used to perform all numerical simulations. A computational model replicating the flow injection-based biosensor system given in Fig. 1 was created. Typical microfluidic channels employed in biosensors have high aspect ratios where side walls are far apart relative to the top/bottom walls allowing microchannel width to be neglected reducing the model to a cross-section through the microchannel. The two dimensional flow cell geometry with height $h = 20$ μM and length $l = 0.5$ mm housing a hydrogel film grafted to the flow cell sensing region (i.e. 200 nm x 0.5 mm sensing domain) was meshed with > 14 k elements. This mesh was optimized until no detectable change was observed in the simulation output and included a higher density of elements at the hydrogel interfacial boundaries arriving at 6762 elements over the hydrogel domain. The incompressible form of the Navier-Stokes equation was used to solve the two-dimensional velocity profile through the channel, assuming steady-state, at constant flow rate and at atmospheric pressure. The velocity at the walls $u_{wall} = 0$, the inlet velocity was variable then solving for the velocity vector field over the full domain

$$\rho u \cdot \nabla u = -\nabla p + \mu \nabla^2 u \quad \text{(S5)}$$

where ρ is the density, p is the pressure and μ is the dynamic viscosity.

The flow velocity vector field was coupled to the steady-state advection/diffusion equation for a dilute species to solve for the analyte concentration field in the bulk flow.

$$\nabla . (-D\nabla c) + u . \Delta c = R \quad \text{(S6)}$$

Here D is the diffusion coefficient, c is analyte concentration and R is a reaction term.
Initially the analyte concentration in the microchannel $c = 0$. At the inlet the initial analyte concentration profile along the microchannel height was defined by multiplying the concentration by a rectangular function to produce a rectangular pulse of injected sample for a given contact time. **P** was assumed to be distributed within the hydrogel domain tethered to the flow cell. The associated reactions within the hydrogel domain between soluble species with **P** distributed within the hydrogel and the formation of non-tethered affinity complexes **AB** were defined as ODEs coupled to the advection/diffusion equation (S6) and are given as

$d\mathbf{A}/dt = -k_a.\mathbf{A}.\mathbf{B} + k_d.\mathbf{AB}$
$d\mathbf{B}/dt = -k_a.\mathbf{A}.\mathbf{B} + k_d.\mathbf{AB} - k_{a'}.\mathbf{P}.\mathbf{B} + k_{d'}.\mathbf{BP}$
$d\mathbf{AB}/dt = k_a.\mathbf{A}.\mathbf{B} - k_d.\mathbf{AB}$
$d\mathbf{P}/dt = -k_{a'}.\mathbf{P}.\mathbf{B} + k_{d'}.\mathbf{BP}$
$d\mathbf{BP}/dt = k_{a'}.\mathbf{P}.\mathbf{B} - k_{d'}.\mathbf{BP}$

where k_a and k_d, are the forward and reverse kinetic interaction constants for the **AB** complex and k_a' and k_d', are the forward and reverse kinetic interaction constants for the **BP** affinity complex. This set of ODEs describe reversible affinity complex formation and formation of an irreversible complex (**AB***) required the following ODEs.

$dA/dt = -k_a.A.B + k_d.AB$
$d\mathbf{B}/dt = -k_a.\mathbf{A}.\mathbf{B} + k_d.\mathbf{AB} - k_{a'}.\mathbf{P}.\mathbf{B} + k_{d'}.\mathbf{BP}$
$d\mathbf{AB}/dt = k_a.\mathbf{A}.\mathbf{B} - k_d.\mathbf{AB} - k_{inact}.\mathbf{AB}.$
$d\mathbf{P}/dt = -k_{a'}.\mathbf{P}.\mathbf{B} + k_{d'}.\mathbf{BP}$
$d\mathbf{BP}/dt = k_{a'}.\mathbf{P}.\mathbf{B} - k_{d'}.\mathbf{BP}$
$d\mathbf{AB^*}/dt = k_{inact}.\mathbf{AB}$

The time-dependent change in analyte accumulation was found from a surface flux balance at the sensing surface where the simulation was performed in time-stepping mode. The accumulation of affinity complex was expressed in terms of an equivalent biosensor response where 1 RU = 10 μg/ml bound species.

Data analysis. Microsoft Excel and Biaevaluation (GE Healthcare Bio-Sciences AB) were employed for data processing. Graphpad Prism version 6 (GraphPad Software, Inc. 7825 Fay Avenue, Suite 230, La Jolla, CA, 92037, USA) was employed for all plots other than Fig. 6 a, b Curve fitting programs enable fitting of binding interaction data to interaction models by nonlinear regression, and the associated statistical methods to confirm goodness of fit and confidence in parameter estimates are well established[S5]. Statistical parameters such as the standard error of the fit (SE) associated with a given parameter returned in the fit were used to report confidence in parameter estimates. The SE is a measure of the information content of the data and specifies the degree to which the curves define the parameter value from the fit. Values < 5 % indicate high confidence and values >10% indicate that the parameter is poorly defined. The goodness of fit between a model curve and an experimental curve is described by χ^2 when the number of data points is high and by a regression coefficient R^2 when the number of values is low. $\%\chi^2$ is the square of the averaged residual response difference expressed as a percentage of maximum response

recorded for the curve set. Typically high quality fits will produce χ^2 values $< 5\%$. Occasionally χ^2 may be within acceptable limits but the fit may remain questionable if residuals are not distributed randomly. Curves generated by numerical simulation follow deterministic algorithms and reproduce without error and therefore do not require replicates.

Global parameter fitting: A fitted parameters is constrained to a single global value over the entire curve set providing a more robust fit to the model. The resulting parameter estimates therefore represent the sum of multiple replicated measurements.

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