Binding Theory
Equations for Affinity and Kinetics Analysis

This technology note summarizes important equations underlying the theory of binding of solute analytes to surface-tethered ligands.

Keywords: Affinity | kinetics | association & dissociation rates k_{on}, k_{off} | affinity (dissociation) constant K_a (K_d) | equilibrium analysis | avidity, bivalent binders

Let A and B be two interacting species in solution which can form a bound product, AB, and let c_A, c_B, c_{AB} be their concentrations in $\frac{mol}{liter} = M$.

$$A + B \rightleftharpoons AB \qquad (1)$$

The time-dependent **rate equations** for the formation and the decay of product c_{AB} are:

$$\frac{dc_{AB}}{dt} = k_{on} \cdot c_A \cdot c_B$$

$$\frac{dc_{AB}}{dt} = -k_{off} \cdot c_{AB} \qquad (2)$$

with the forward reaction rate constant k_{on} and reverse reaction rate constant k_{off}.

k_{on} is also called the on-rate or association rate, and k_{off} is the off-rate or dissociation rate. Note that k_{on} and k_{off} have different units:

$$[k_{on}] = M^{-1}s^{-1} \qquad [k_{off}] = s^{-1}$$

Equilibrium

In equilibrium the sum of all time-derivatives is zero,
$\Sigma\, dc_{AB}/dt = 0 \rightarrow k_{on}c_A c_B = k_{off}c_{AB}$,
which yields a fundamental chemical equation, the **law-of-mass-action** in **solution**:

$$K_a = \frac{1}{K_d} \equiv \frac{k_{on}}{k_{off}} = \frac{c_{AB}}{c_A \cdot c_B}$$
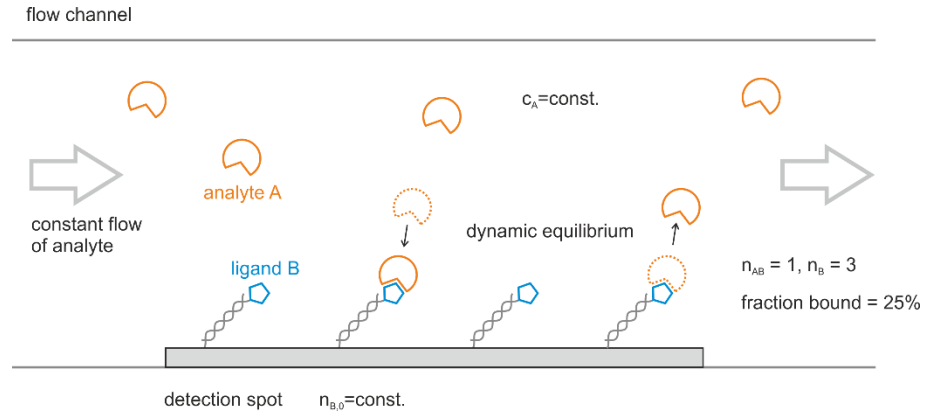
$$\qquad (3)$$

The affinity constant K_a links the concentration of bound molecules to the concentrations of free reactants and hence gauges the strength of the interaction.

$$\text{or} \quad c_{AB} = K_a \cdot c_A \cdot c_B$$

It is often practicable to consider the dissociation constant K_d, because it has the unit of a concentration, and thus can be compared to the reactant concentrations.

$$[K_a] = M^{-1} \qquad [K_d] = M$$

Figure 1 | Dynamic equilibrium on a switchSENSE biosensor, the example shows a fraction bound of 25% ▶

flow channel

c_A=const.

analyte A

constant flow of analyte

dynamic equilibrium

ligand B

$n_{AB} = 1$, $n_B = 3$

fraction bound = 25%

detection spot $n_{B,0}$=const.

In solution, c_{AB} depends on the concentrations of both reactants c_A and c_B, but for surface biosensors the law-of-mass-action simplifies, because the total number (surface density) of capture molecules which are immobilized on the biosensor surface, $n_{B,0}$, is constant. In eq. 4, n_B denotes the number of free binding sites on the surface and n_{AB} is the number of binding sites occupied with analyte A. While n_B and n_{AB} correspond to c_B and c_{AB} in the solution case, we use a different notation to emphasize the different nature of solute reactants (c) and surface-immobilized reactants (n).

In an ideal biosensing experiment, the number of solute reactants should be in large excess of the number of surface binding sites so that effectively c does not change when molecules adsorb from the solution onto the surface. This simplifies matters to a great extent and usually is accomplished by providing a constant flow of fresh analyte solution to the sensor.

An essential quantity in a biosensing experiment is the '**fraction bound**' (f) value, because it is proportional to the biosensor signal.

The fraction bound is defined as the number of occupied binding sites on the detection spot (n_{AB}) divided by the total number of binding sites ($n_{B,0}$). The fraction bound is 0% for a pristine sensor and reaches 100% when the sensor surface is fully saturated with analyte molecules.

Inserting (4) into (3) and rearranging according to (5) yields the equivalent of the **law-of-mass-action** for **surface biosensors**:

$$n_{B,0} = n_B + n_{AB} = const.$$

(4)

$$n_B \sim c_B \qquad n_{AB} \sim c_{AB}$$

$$fraction\ bound \equiv \frac{n_{AB}}{n_{B,0}} \propto signal \qquad (5)$$

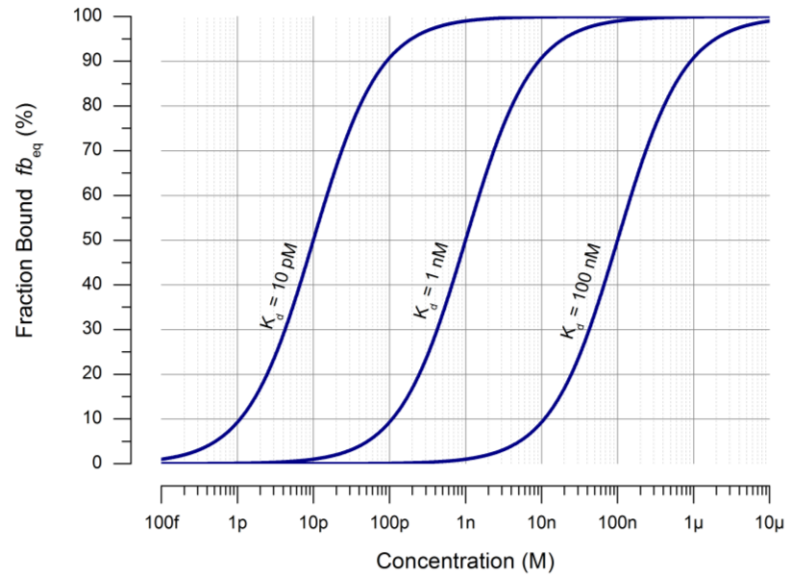$fraction\ bound\ in\ equilibrium$:

$$f_{eq}(c) = \frac{c}{c + K_d}$$

(6)

Eq. 6 pertains to the equilibrium state ($t \to \infty$). It corresponds to the Langmuir isotherm, which has been derived for the adsorption of gas molecules onto surfaces. Compared to the law-of-mass-action in solution, the equation is simpler; it only depends on the analyte concentration c and the equilibrium dissociation constant K_d.

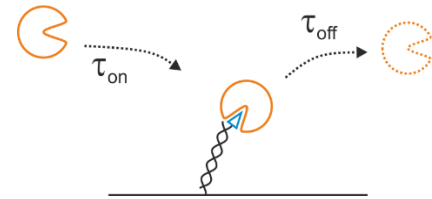(Note that $c = c_A$; the index A is omitted for simplicity in the following.)

Figure 2 | Fraction bound calculated with eq. 6. The fraction bound function is a sigmoidal curve centered around K_d, i.e., if the analyte concentration is equal to the dissociation constant, 50% of the binding sites on the surface are occupied with analyte, $f_{eq}(c = K_d) = 0.5$. Note that f_{eq} varies significantly only if c is within plus/minus two orders of magnitude from the K_d value. For low concentrations, $c < 0.01 \times K_d$, less than 1% of the binding sites are occupied with analyte, while for high concentrations, $c > 100 \times K_d$, more than 99% of the sensor's binding capacity is saturated. ▶

Binding kinetics

When the concentration of analyte molecules above the sensor changes, a new equilibrium of bound (unbound) ligands adjusts on the surface . Binding kinetics may be analyzed from real-time data by integrating the rate equations (2).

Figure 3 ▶

For the **association phase** at a given analyte concentration c the solution of (2) is

$$f(t,c) = f_{eq}(c) \cdot \left[1 - exp\{-k_{on}^{obs} \cdot t\}\right] \qquad (7)$$

with the observable on-rate k_{on}^{obs}

$$k_{on}^{obs} = c \cdot k_{on} + k_{off} \qquad (8)$$

The signal approaches its terminal value f_{eq} in an exponential manner with the characteristic time constant τ_{on}^{obs}. As f_{eq} depends on the analyte concentration, so does the observable signal change. In practice, this makes measurements at low concentrations ($c \ll K_d$) difficult because association kinetics are slow and the attainable signal change is small.

$$\tau_{on}^{obs} = 1/k_{on}^{obs} \qquad (9)$$

Note that the observable rate constant k_{on}^{obs} depends not only on c and the intrinsic association rate k_{on}, but also on the off-rate k_{off}. This has the somewhat counterintuitive consequence that the kinetics during the association phase are influenced by the dissociation rate, especially if the dissociation rate is very high. For low dissociation rates or high analyte concentrations ($c \cdot k_{on} \gg k_{off}$), however, the dissociation rate may be neglected during the association phase and $k_{on}^{obs} \approx c \cdot k_{on}$.

The **dissociation phase** is measured by removing the analyte solution above the sensor and exchanging it with pure running buffer ($c = 0$). It is solely controlled by the dissociation rate constant k_{off} and the dissociation time constant τ_{off}, respectively.

$$f(t) = a \cdot exp\{-k_{off} \cdot t\} \qquad (10)$$

$$\tau_{off} = 1/k_{off} \qquad (11)$$

The observable dissociation time constant τ_{off} does not depend on the conditions during the association phase. Regardless of the used analyte concentration and the duration of the association phase (the actual saturation state of the sensor), the dissociation phase always features the same k_{off} value. The prefactor a (amplitude) corresponds to the fraction bound right before the analyte solution is exchanged with running buffer.
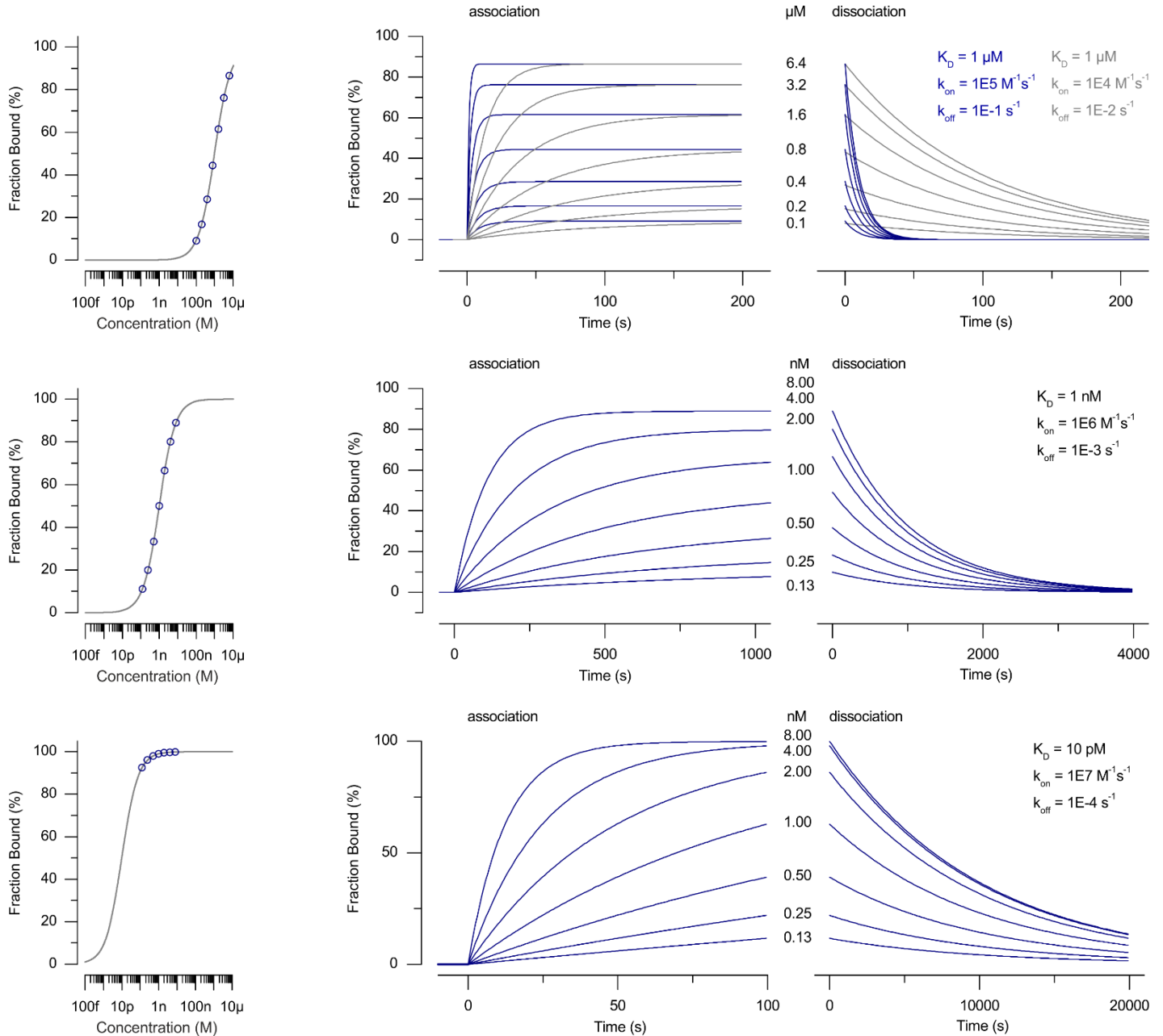

Figure 4 | Exemplary solutions to equations (6), (7), and (10) for $K_D = 1\,\mu M$ (top, e.g. a small molecule–protein interaction), $K_D = 1\,nM$ (middle, e.g. a protein–protein interaction), $K_D = 10\,pM$ (bottom, e.g. a high affinity antibody − protein interaction). Concentrations are listed between the kinetic curves and also indicated in the fraction bound plot.

Bi-phasic association/dissociation kinetics

So far we have considered a mono-phasic association and dissociation behavior, which pertains to the simplest case where one type of analyte interacts with one type of surface-bound ligand, involving only one type of binding site, cf. Fig. 2.

However, more complex situations lead to a **bi-phasic or multi-phasic dissociation/association behavior**, like the examples depicted in Figure 5.

bivalent analyte with interlinking capability

mixture of analytes with binding sites for same ligand

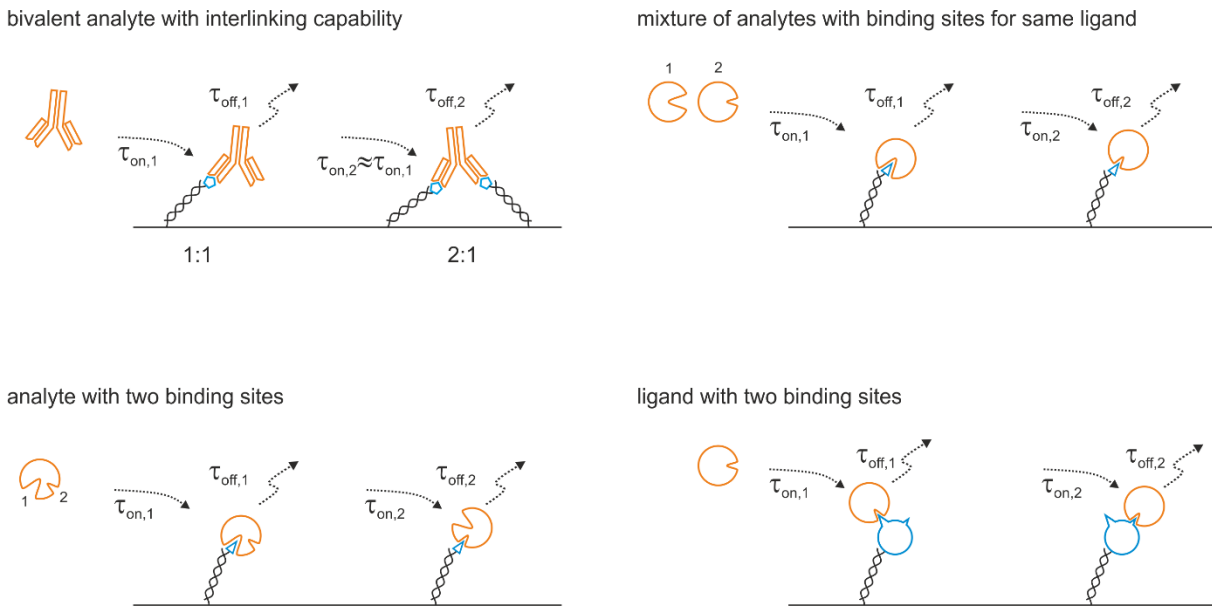analyte with two binding sites

ligand with two binding sites

Figure 5 | Complex binding situations which lead to a bi-phasic dissociation behavior and a mono- or bi-phasic association behavior, respectively.

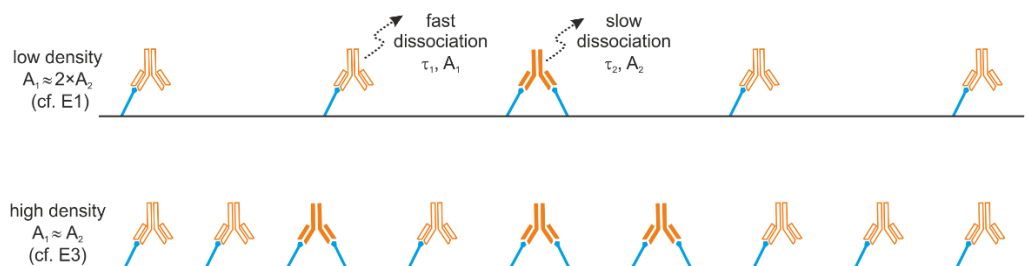Equation (10) can be expanded to account for more than one dissociation process:

$$f(t) = \begin{aligned} & a_1 \cdot exp\{-k_{off,1} \cdot t\} \\ & +a_2 \cdot exp\{-k_{off,2} \cdot t\} \\ & + \cdots \end{aligned} \tag{12}$$

The amplitudes $a_1, a_2, \ldots$ reflect the respective contributions of the different dissociating species to the overall dissociation curve.
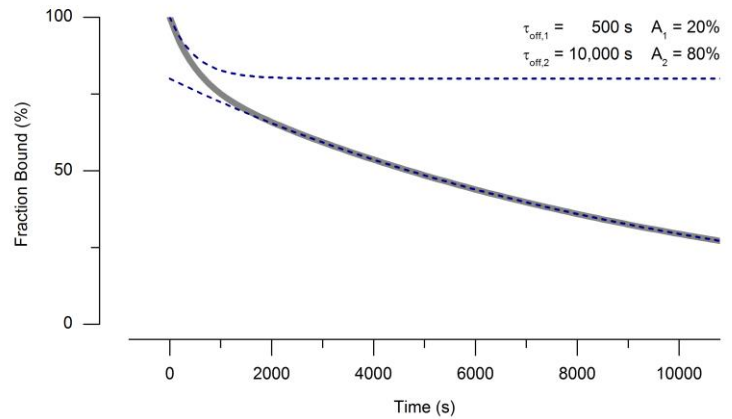
$$a_1 + a_2 + \cdots = 100\% \tag{13}$$

Bivalent antibodies (cf. Fig. 5 top left) are exemplary analytes which exhibit bi-phasic dissociation behavior: antibodies which bind to antigen-probes via one arm only feature fast dissociation rates (high $k_{off,1}$ ~ short $\tau_{off,1}$), while antibodies which are bound to two antigen-modified probes by interlinking them feature a slow dissociation rate (low $k_{off,2}$ ~ long $\tau_{off,2}$).

Figure 5 | Schematic illustration of bivalent antibodies dissociating from detection spots with a low density (top) and a high density (bottom) of antigen-modified probes. ▶
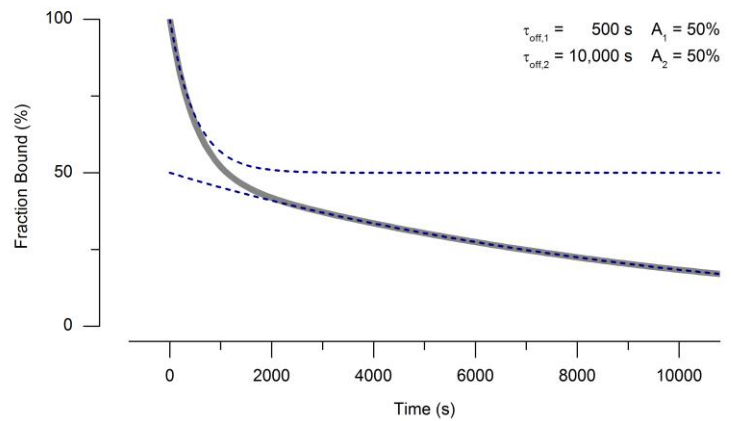

To test whether a biphasic dissociation behavior stems from bivalent analytes like antibodies, it is advisable to investigate antigen layers with different densities. The relative contribution (amplitude) a_2 of the slow dissociation rate $k_{off,2}$ is expected to drease with

decreasing antigen density, because the probability for interlinking becomes lower.
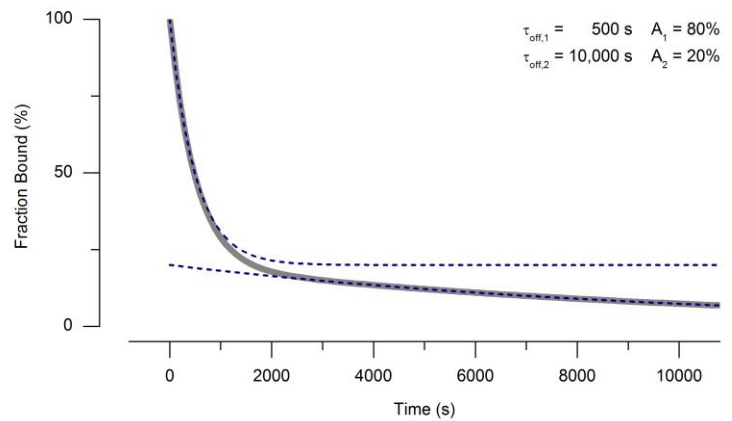

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Figure 6 | Calculated biphasic dissociation curves (gray solid lines), which are superpositions of two exponential functions (dashed blue lines) with time constants differing by a factor of 20. The three cases exemplify different relative amplitudes:

$a_1 = 20\% \quad a_2 = 80\%$
$a_1 = 50\% \quad a_2 = 50\%$
$a_1 = 80\% \quad a_2 = 20\%$
(from top to bottom). ▶

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