EXPERIMENTAL DETERMINATION OF Kd

TECH. THAT DO NOT REQUIRE SEPARATION OF BOUND FROM FREE LIGAND

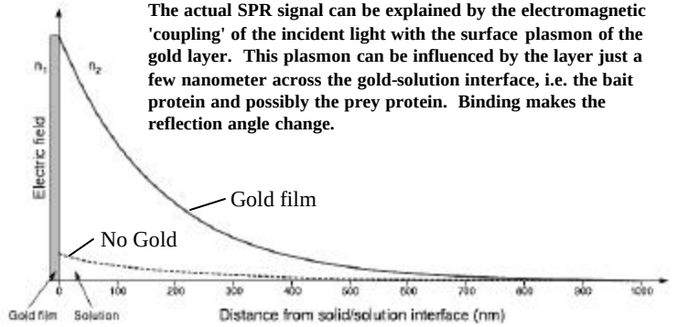
- ***Equilibrium dialysis*** - Place M in a dialysis bag and dialyse against a solution containing a ligand whose concentration can be determined using radioisotopic or spectroscopic techniques.

- ***Fluorescence spectroscopy*** - Find a ligand whose absorbance or fluorescence spectra changes when bound to M. Alternatively, monitor a group on M whose absorbance or fluorescence spectra changes when bound to L.

- ***ITC - Isothermal Titration Calorimetry*** – Measure small, incremental heats (Dq) of reaction during binding titration. Obtain DH, n and Keq, then calc DG and DS.

- ***Kinetic (higher tech) methods:***
  - ***SPR – Surface Plasmon Resonance*** k_{on} / k_{off}
  - ***Fast Kinetics*** – rate constants

SPR - The need for Gold



The actual SPR signal can be explained by the electromagnetic 'coupling' of the incident light with the surface plasmon of the gold layer. This plasmon can be influenced by the layer just a few nanometer across the gold-solution interface, i.e. the bait protein and possibly the prey protein. Binding makes the reflection angle change.

Binding - SPR or BIA

"**The secret of life is molecular recognition**"

"Binding is the first step necessary for a biological response"

Biacore 's SPR technology: label-free technology for *monitoring biomolecular interactions as they occur*.

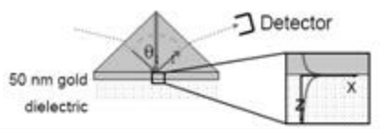
The detection principle relies on surface plasmon resonance (SPR), an electron charge density wave phenomenon that arises at the surface of a metallic film when light is reflected at the film under specific conditions.

The resonance is a result of energy and momentum being *transformed* from incident photons into surface plasmons, and is sensitive to the refractive index of the medium on the <u>opposite side</u> of the film from the reflected light.

Hackert – CH370

Note: Many of these figures/notes were taken from on-line resources from Biacore

Plasmons & SPR "angle"



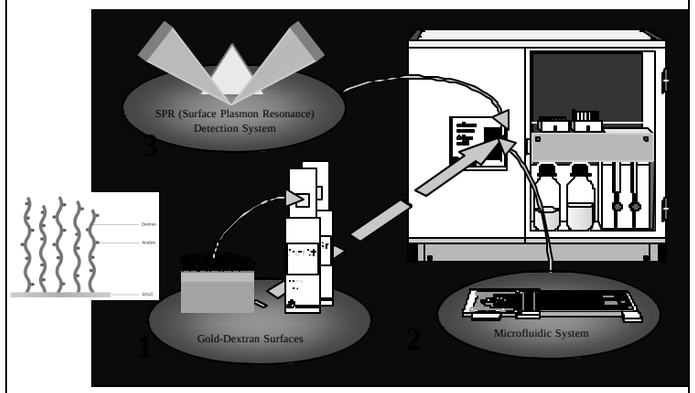
Measure reflected (polarized) light as function of angle.

At a certain "Magic Angle" light is not reflected ("total internal reflection") but interacts with free electrons in gold to form a resonant energy wave – or surface plasmon.

Plasmon – A plasmon is a collective oscillation of the conduction electrons in a metal - a quasiparticle that can be regarded as a hybrid of the conducting electrons and the photon.

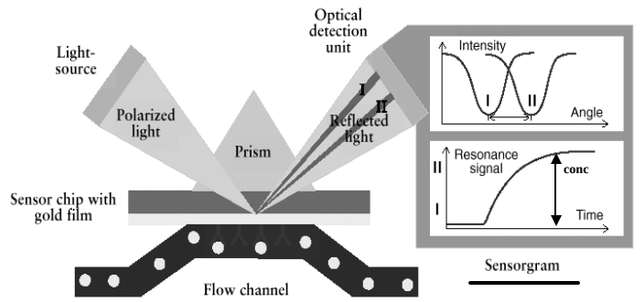
Angle is sensitive to refractive index of dielectric which varies with concentration of molecules on the other side of gold layer!

Three Corner Stones of Biacore Technology

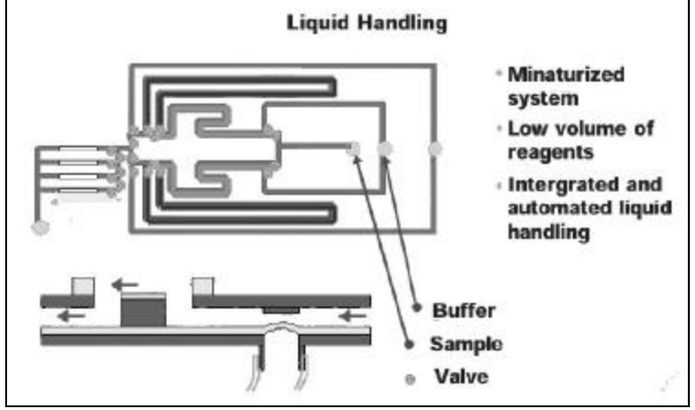


3. Surface Plasmon Resonance Detection: Biomolecular Binding in Real Time

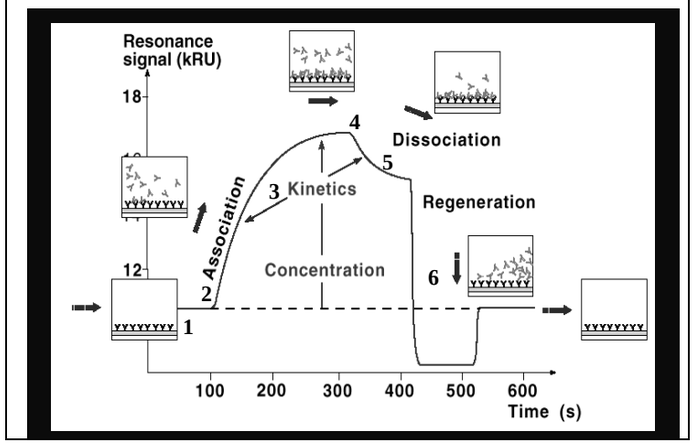
Principle of Detection



2. Integrated micro Fluidics Cartridges (IFC)

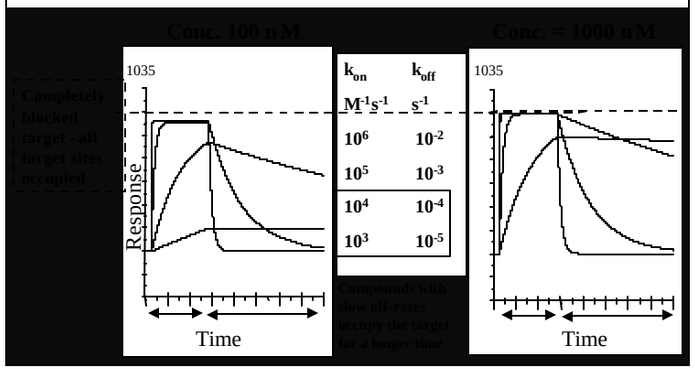


The Sensorgram is Information Rich



Same affinity but different kinetics

- All 4 compounds have the **same affinity** K_D = 10 nM = 10^{-8}M
- The binding **kinetic constants vary by 4 orders** of magnitude



Chemical Kinetics: the study of the rate of reactions

rate measurements + dependence of experimental conditions

Mechanism: Explain what the molecules are doing / a set of reactions showing how molecules collide and make and break bonds.

For ***one stoichiometric reaction***, there are ***many mechanisms.***

Principle of microscopic reversibility

Rate Law / Order of Reaction

Sucrose + water ---- (H+) → fructose + glucose

Measuring rate data: [ ] vs. time / "quenching" if time to measure is long compared to rate of reaction. → "Quenched-flow" apparatus

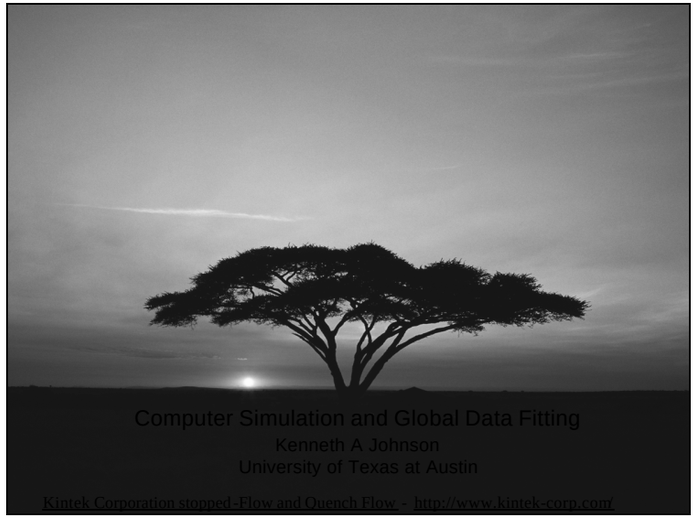
Summary

- **SPR detects binding events as changes in mass at the chip surface**
- **Real-time kinetic measurements**
- **Qualitative rankings**
- **Measurement of *active* concentration**
- **Information about structure-activity relationships**
- **Low volumes of precious samples needed**

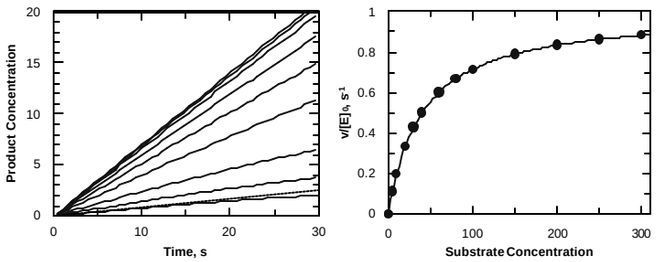
BUT !!! -

SPR is not a true solution method (vs. ITC)

Attaching receptor to surface can influence binding properties.



Conventional Steady-State Kinetics

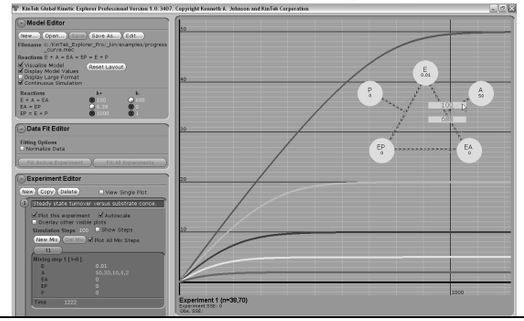


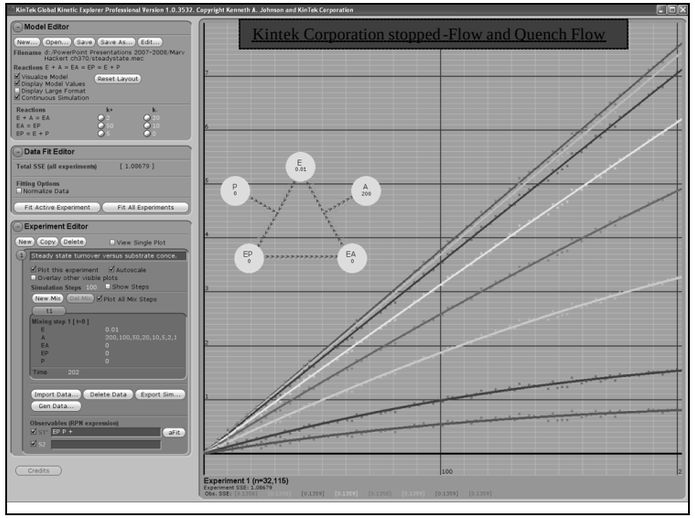
1. Measure initial rate
   a. Restrict data collection to first 10% of reaction
   b. If there is curvature, fit to polynomial to get initial rate
2. Plot rate versus concentration
3. Fit secondary plot to extract k_{cat} and K_m

Full Time-course (Progress Curve) kinetics

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} E + P$$

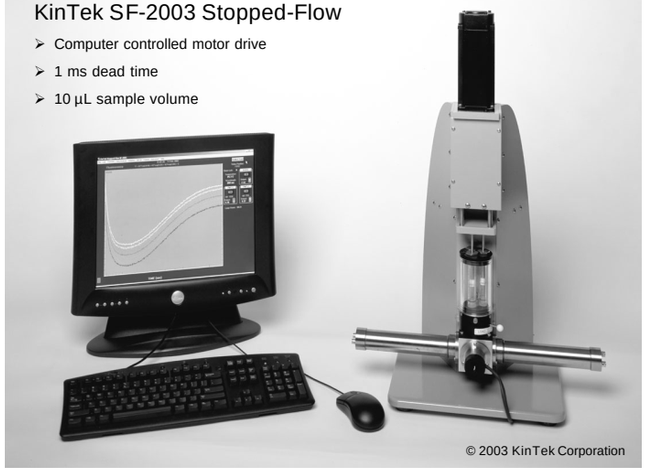
Follow reaction until reaction approaches equilibrium
Decreasing rate of turnover is due to decreasing [S] and increasing [P]
Data can be fit directly by simulation to extract k_{cat} and K_m



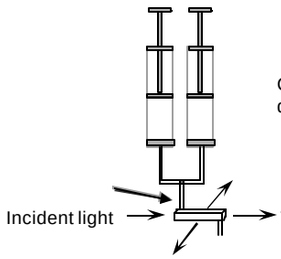


KinTek SF-2003 Stopped-Flow

- Computer controlled motor drive
- 1 ms dead time
- 10 µL sample volume



KinTek Stopped-Flow



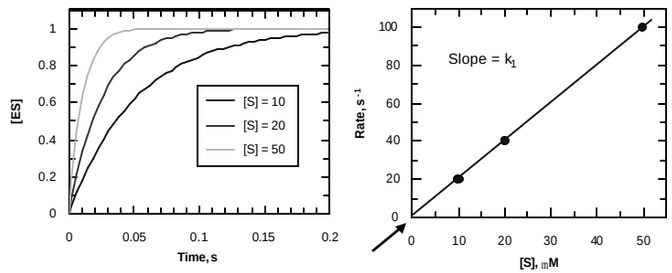
Computer-controlled drive syringes

Fluorescence or Light scattering

- 30 µl per shot
- 1 msec dead time
- 10 µl observation cell

Concentration dependence of binding rate

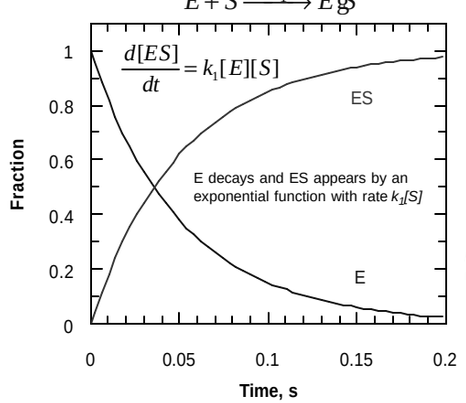
$$k_{obs} = k_1[S]$$



Kinetics of irreversible substrate binding

$$E + S \xrightarrow{k_1} EgS$$

$$\frac{d[ES]}{dt} = k_1[E][S]$$



k_1 = 2 µM^{-1}s^{-1}

[S] = 10 µM

k_1[S] = 20 s^{-1}

$t_{1/2}$ = (ln 2)/k

= 34.6 msec

k_1 is a second order rate constant, units M^{-1}s^{-1}

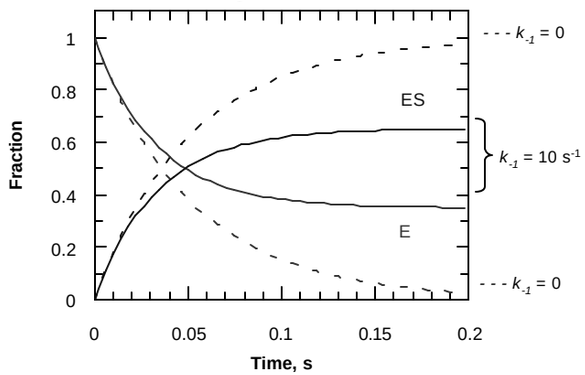
$k_1[S]$ is the pseudo-first order rate constant, unit s^{-1}

Kinetics of reversible binding

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EgS$$

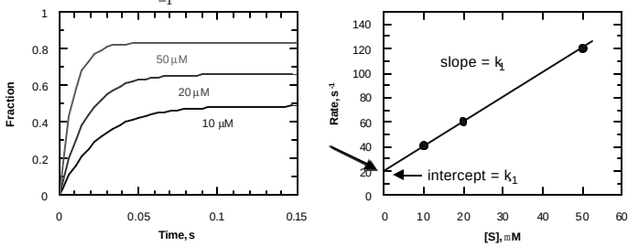
$$k_{obs} = k_1[S] + k_{-1}$$

Observed rate is the sum of forward and reverse rates.



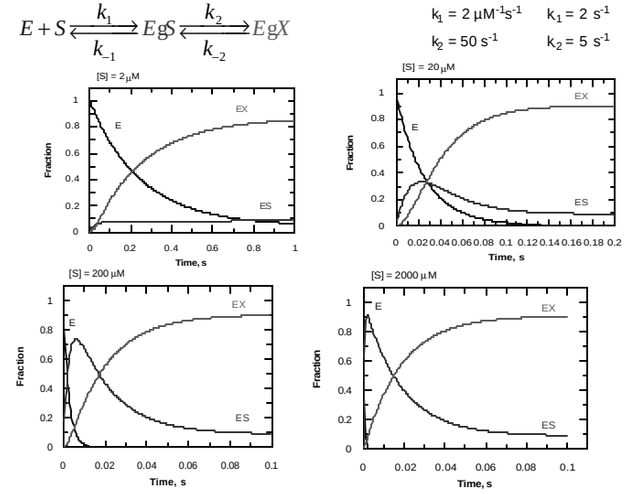
Concentration dependence of binding rate

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EgS \qquad k_{obs} = k_1[S] + k_{-1}$$



NOTE: increase in amplitude and rate as a function of increasing [S]

One experiment can serve to define k_1, k_{-1} and K for S binding.



Kinetics of substrate binding: *Two-steps, four rates*

C. Complete solution $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EgS \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EgX$

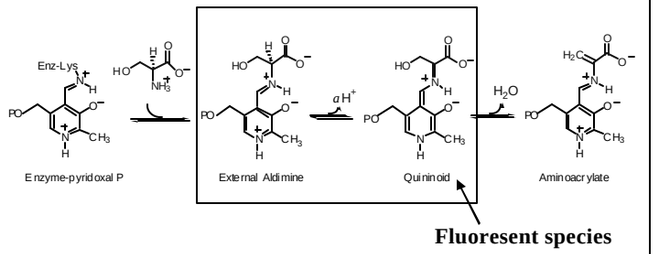
Each species follows a double exponential

$$[E]_i / [E]_0 = A_1 e^{-l_1 t} + A_2 e^{-l_2 t} + C$$

with rates of: $l_1 \approx k_1[S] + k_{-1} + k_2 + k_{-2}$

$$l_2 \approx \frac{k_1[S](k_2 + k_{-2}) + k_{-1}k_{-2}}{k_1[S] + k_{-1} + k_2 + k_{-2}}$$

Reaction with serine with pyridoxal phosphate

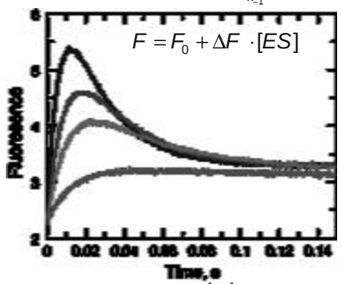


$$E + Ser \underset{20s^{-1}}{\overset{0.135mM^{-1}s^{-1}}{\rightleftharpoons}} E{\sim}Ser \underset{10s^{-1}}{\overset{45s^{-1}}{\rightleftharpoons}} E{\sim}X$$

Tryptophan Synthase

Global Data Fitting based upon Simulation

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EX$$



[Serine] µM

1000

500

300

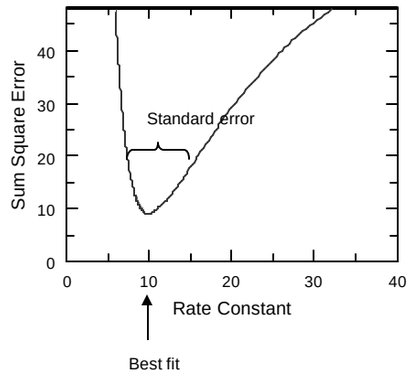
100

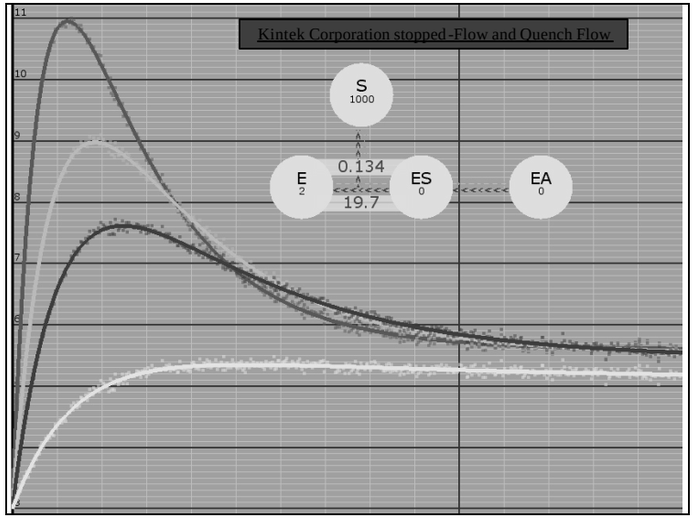
$$E + S \underset{20\,s^{-1}}{\overset{0.135\,mM^{-1}s^{-1}}{\rightleftharpoons}} E\sim S \underset{10\,s^{-1}}{\overset{45\,s^{-1}}{\rightleftharpoons}} E\sim X$$

Fit data directly to the model, get 4 rate constants and two fluorescence output factors.

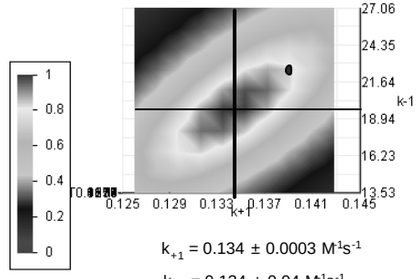
Anderson, K.A., Miles, E. W. and Johnson K. A. (1991) J. Biol. Chem 266, 8020-8033

Nonlinear Regression: minimize sum square error





Confidence Contour from Data Fitting

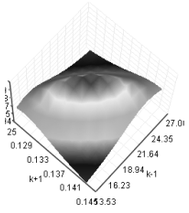


$k_{+1} = 0.134 \pm 0.0003\ M^{-1}s^{-1}$

$k_{+1} = 0.134 \pm 0.04\ M^{-1}s^{-1}$

$k_{-1} = 19.7 \pm 0.09\ s^{-1}$

$k_{-1} = 19.7 \pm 3\ s^{-1}$



FIT = $SSE_{min}/SSE_{x,y}$