Kinetic Separation:

a conceptual platform for development of solution-based kinetic affinity methods

(an Analytical Swiss Army Knife)

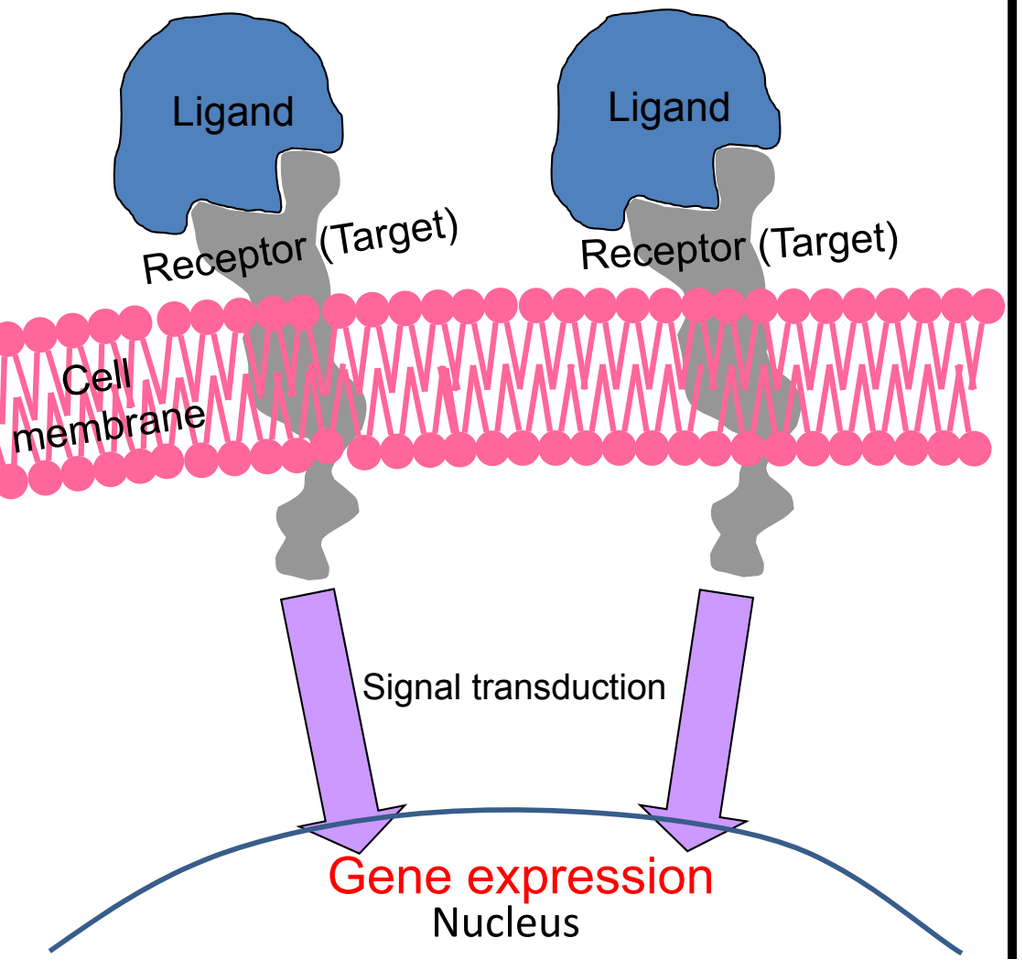
Part 1

Affinity methods and Kinetic Separation in their context

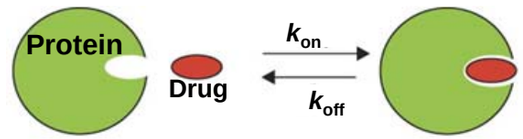
Affinity interactions: Specific strong reversible binding of molecules

$$\text{Target + Ligand} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{Complex} \quad \text{or} \quad \text{T + L} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{C} \qquad K_d = \frac{[T]_{eq}[L]_{eq}}{[C]_{eq}} = \frac{k_{off}}{k_{on}}$$

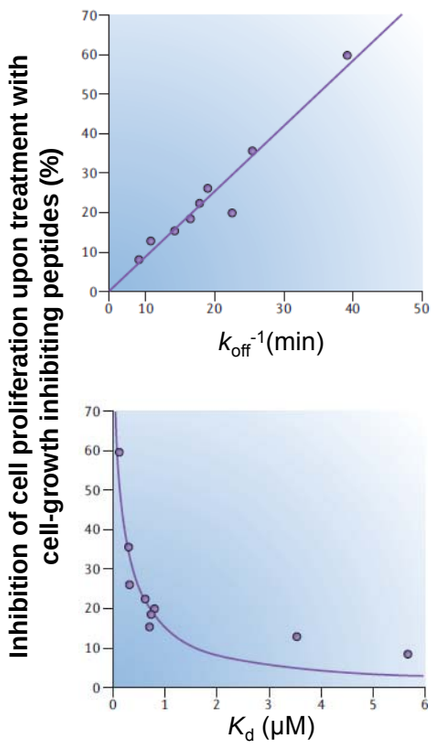
1. Reversible binding drives regulatory processes in biology



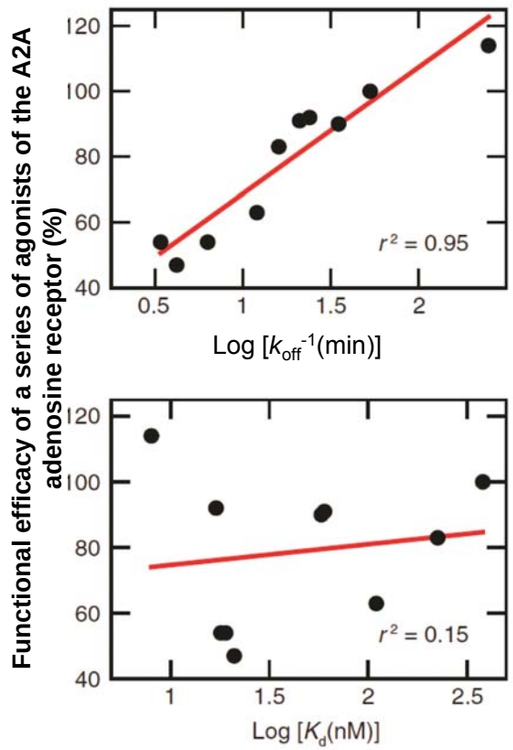
2. Modern drugs are develop to reversibly bind their therapeutic targets



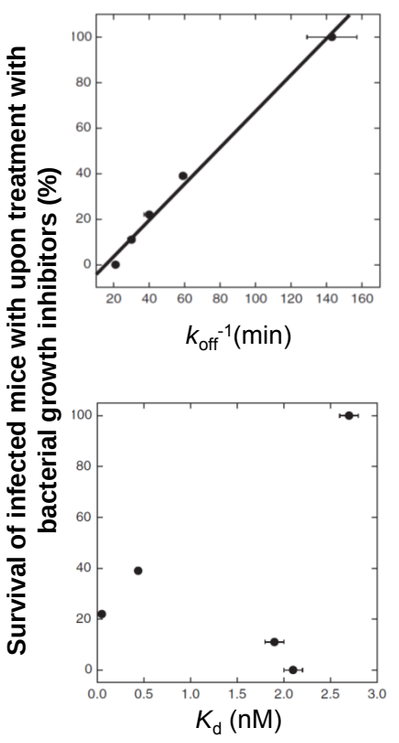
- K_d has been typically used to rank drug leads
- Drugs with the same K_d but different k_{on} and k_{off} may have different efficacies
- It is important to know k_{on} and k_{off}



Nature Rev | Drug Discovery 2007, 5, 730



Drug Discovery Today 2013 ,18 , 456

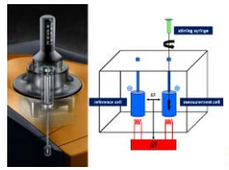


ACS Chem Biol 2009, 4, 221

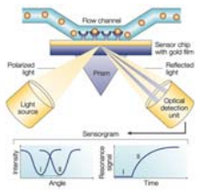
Affinity methods

Finding K_d, k_{on}, k_{off}

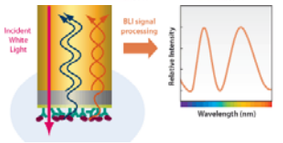
- Calorimetry (K_d only)



- Optical methods:
  - surface plasmon resonance



  - biolayer interferometry



Quantitative detection using affinity probes

- Immunoassays (mainly for proteins)
- Hybridization assays (for DNA and RNA)

Selection of affinity probes and drug candidates from complex mixtures

- Affinity chromatography
- Filtration

Limitation of conventional affinity methods:

1. Different concepts 2. Different instruments 3. Different mathematics

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<u>Our goal</u> is to develop a multi-faceted approach which facilitates three applications:

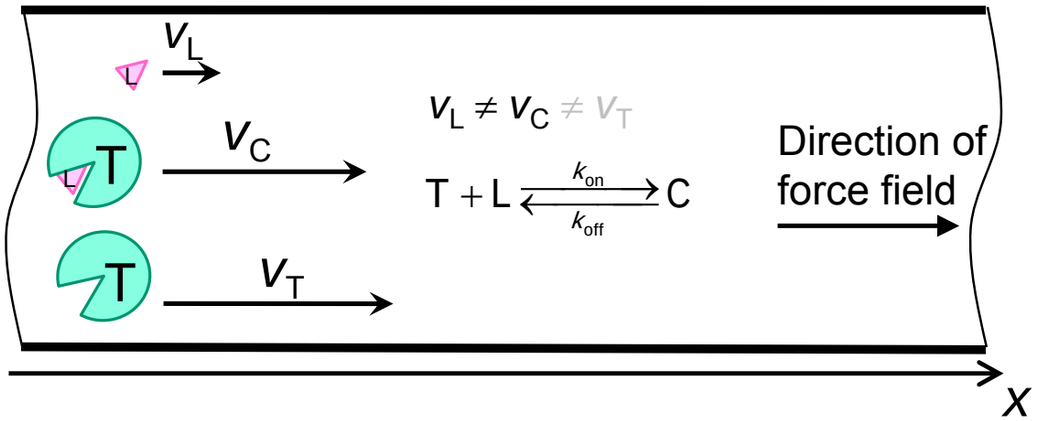
- Finding k_{on}, k_{off}, K_d
- Quantitative detection
- Selection of affinity probes and drug candidates

based on:

1. Single concept 2. Single instrument 3. Single mathematics

Our approach: Kinetic Separation

<u>Definition:</u> Kinetic Separation is separation of interacting species in a narrow tube without immobilization of T or L



Two major processes

1. Reversible binding of **T** to **L**
2. Migration of **T**, **L**, and **C** with different velocities

Mass transfer is described by a system of differential equations:

$$\frac{\partial [T]}{\partial t} + v_T \frac{\partial [T]}{\partial x} = -k_{on}[T][L] + k_{off}[C]$$

$$\frac{\partial [L]}{\partial t} + v_L \frac{\partial [L]}{\partial x} = -k_{on}[T][L] + k_{off}[C]$$

$$\frac{\partial [C]}{\partial t} + v_C \frac{\partial [C]}{\partial x} = -k_{off}[C] + k_{on}[T][L]$$

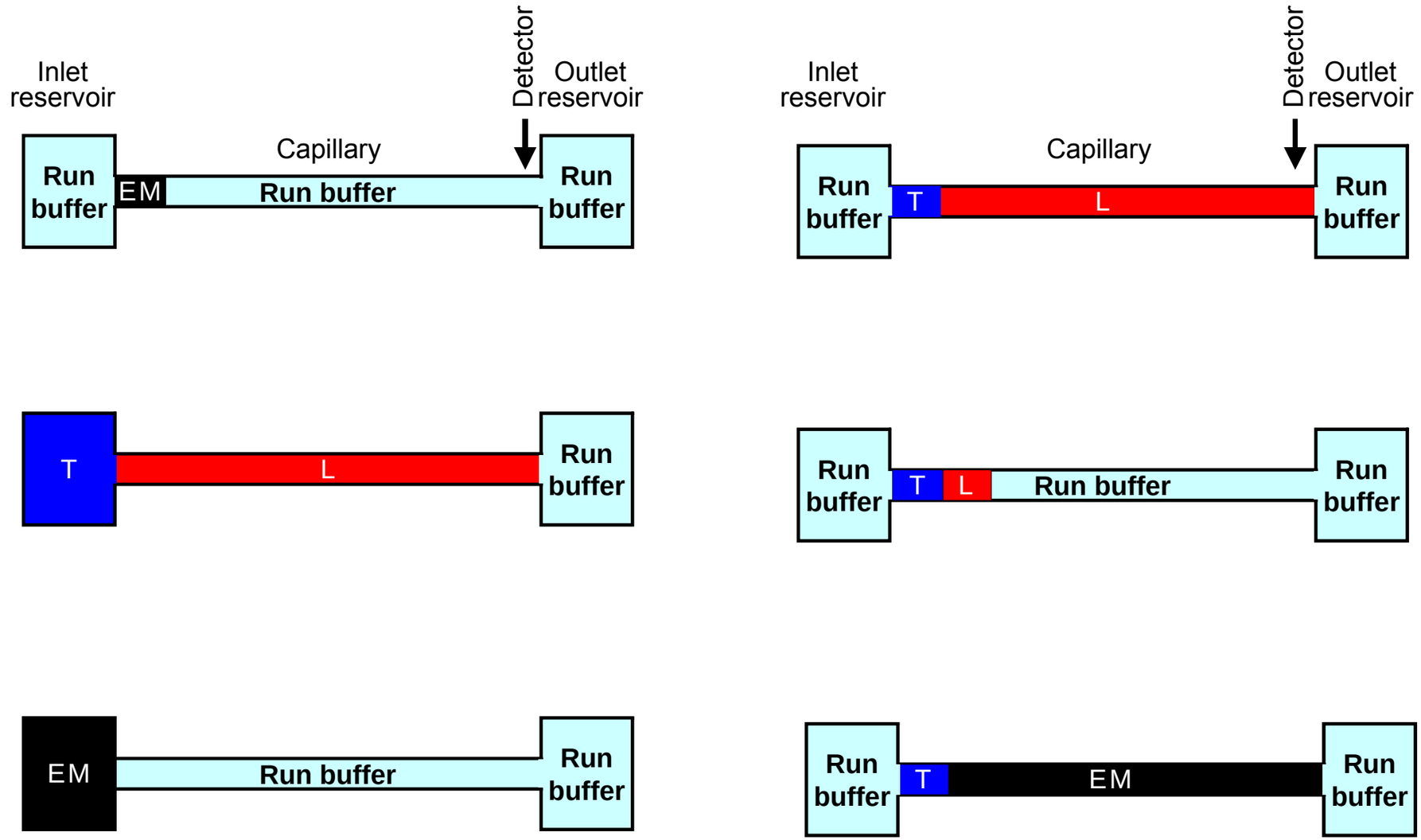
k_{on} and k_{off} can be found by solving these equations for experimental [L](t) and known velocities

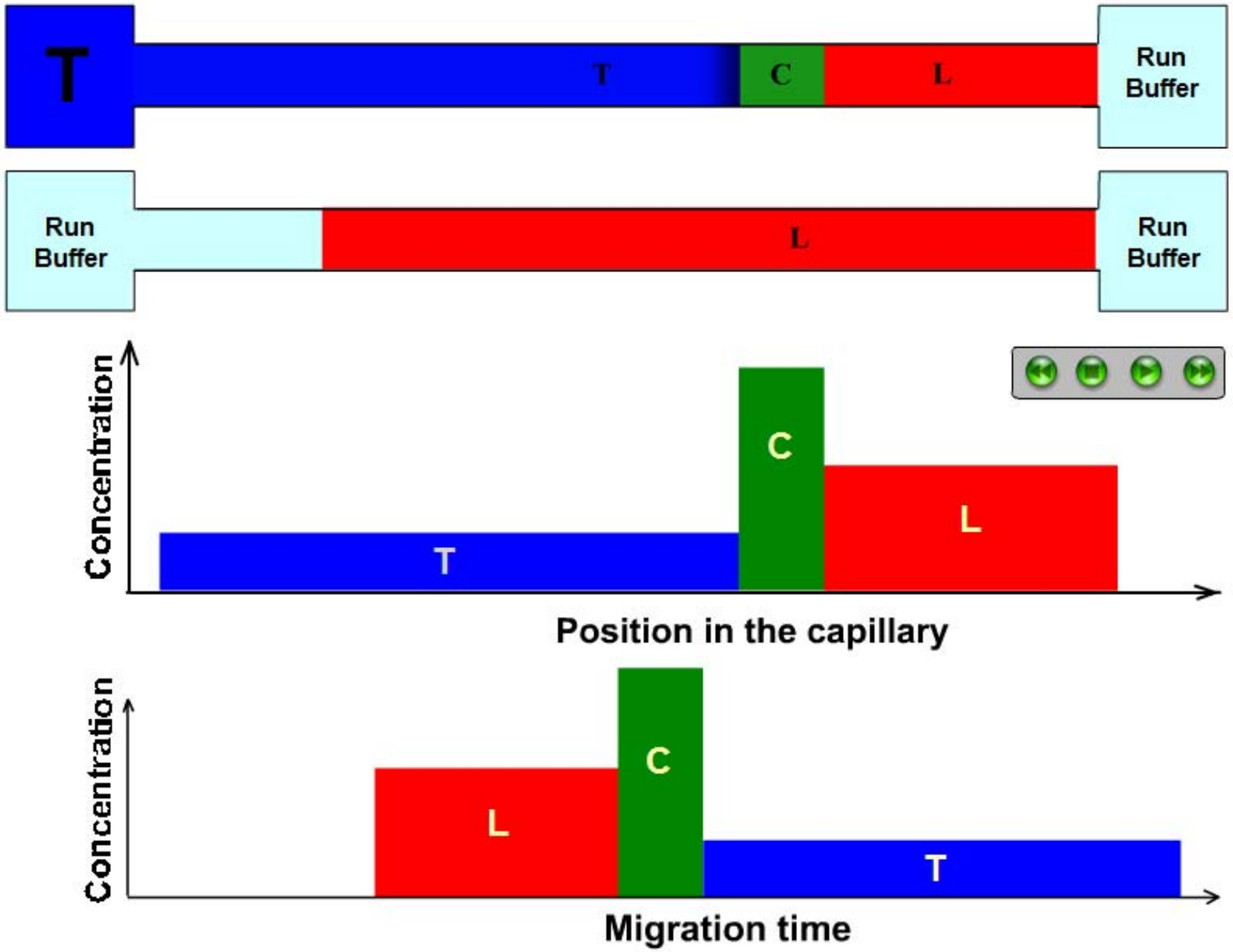
- Qualitatively unique sets of initial and boundary conditions define different methods of Kinetic Separation

- **E**quilibrium **M**ixture = **EM** = L_{eq} + C_{eq} + T_{eq}

$$K_d = \frac{[T]_{eq}[L]_{eq}}{[C]_{eq}}$$

Schematic representation of initial and boundary conditions in Kinetic Separation




T
T
C
L
Run Buffer
Run Buffer
L
Run Buffer
Concentration
C
L
T
Position in the capillary
Concentration
L
C
T
Migration time

Separation and detection approaches suitable for Kinetic Analysis

Separation

Requirements:
1. Different velocities of **L** and **C** (and T): $v_L \neq v_C \neq v_T$
2. Negligible influence of separation on k_{on} and k_{off}

Suitable approaches:

~~1. Affinity chromatography~~ (affects k_{on} and k_{off})
2. ~~Reversed phase chromatography~~ (affects k_{on} and k_{off})
3. ~~Ion exchange chromatography~~ (affects k_{on} and k_{off})
4. Kinetic Capillary Electrophoresis (KCE)
5. Sedimentation (different gravity)
6. Kinetic Size-Exclusion Chromatography (KSEC)

Detection

Requirements:
1. Negligible influence of detection on k_{on} and k_{off}
2. nM sensitivity for studying complexes with nM K_d values

Suitable approaches:

1. Fluorescence labeling (can affect k_{on} and k_{off} of protein-small molecule binding)
2. ~~Label-free optical detection~~ (low sensitivity)
3. Mass Spectrometry (MS)

There are 4 practical kinetic separation options:

KCE-Fluor. and KSEC-Fluor.

KCE-MS and KSEC-MS

Proven applications of Kinetic Separation

1. Finding k_{on}, k_{off}, and K_d for protein-ligand binding
2. Finding ΔH and ΔS of protein-ligand binding
3. Affinity analyses of proteins using DNA aptamers as affinity probes
4. Selection of "smart" ligands (ligands with desirable range of k_{off} or K_d)

All developed methods have been fully documented and published or patented for end users

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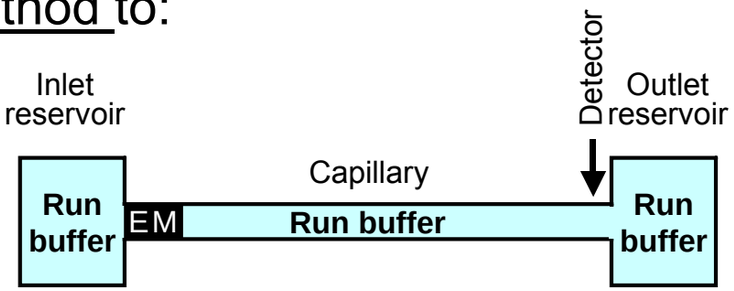
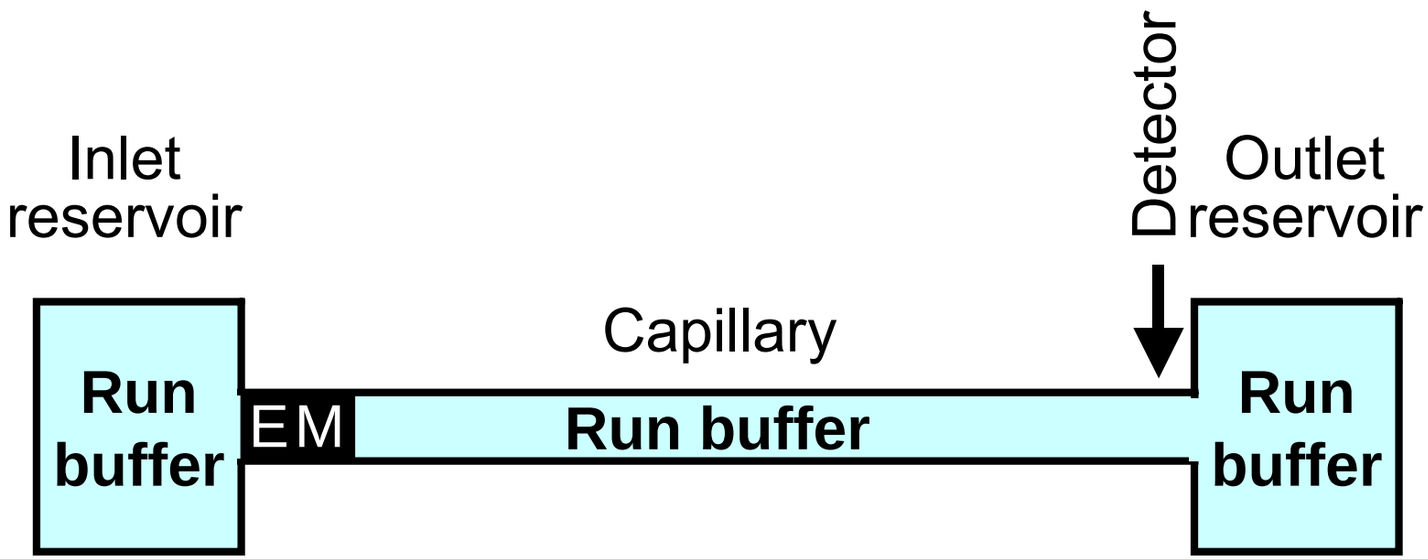
Subject of this lecture is application of a single Kinetic Separation method to:

(i) selection of ligands from combinatorial libraries
(ii) kinetic characterization of target-ligand binding
(iii) calibration-free affinity analysis of target using ligand as affinity probe



Part 2

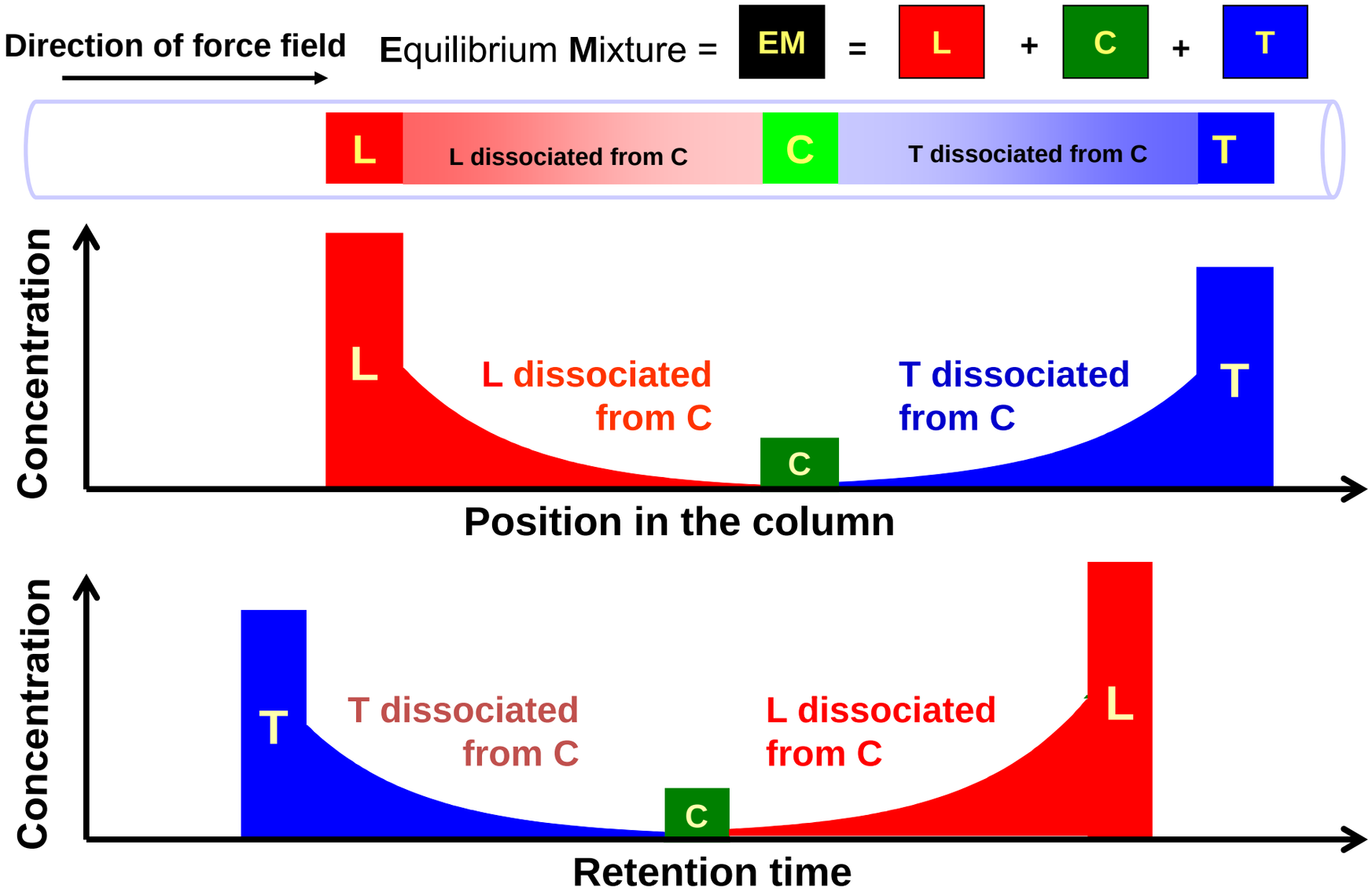
Conceptual explanation of one Kinetic Separation method and its applications



$$EM = L_{eq} + C_{eq} + T_{eq}$$

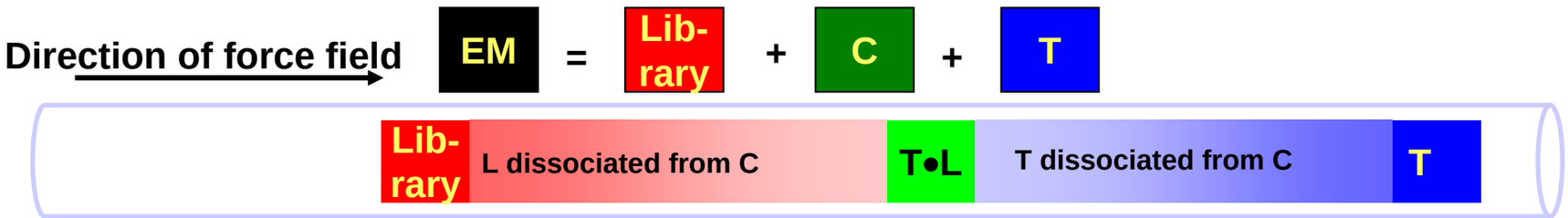
$$K_d = \frac{[T]_{eq}[L]_{eq}}{[C]_{eq}}$$

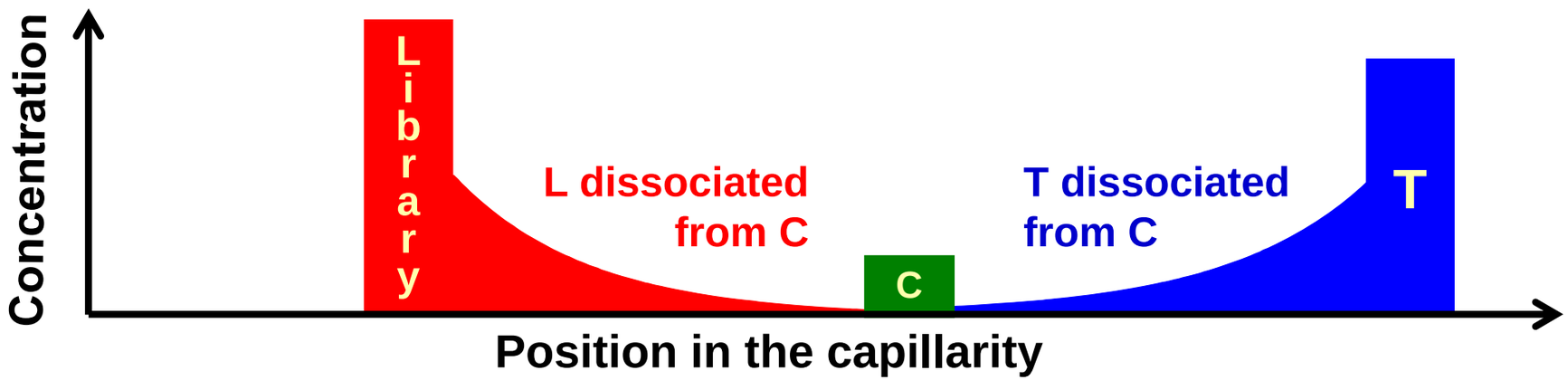
Migration of zones in Kinetic Separation

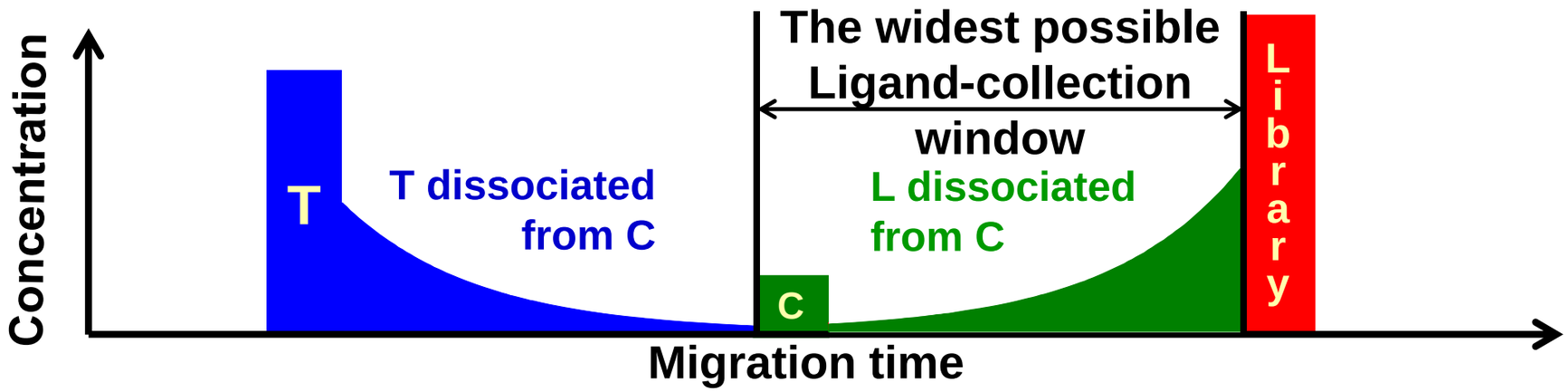


Concept of Kinetic-Separation-based selection of naive ligands

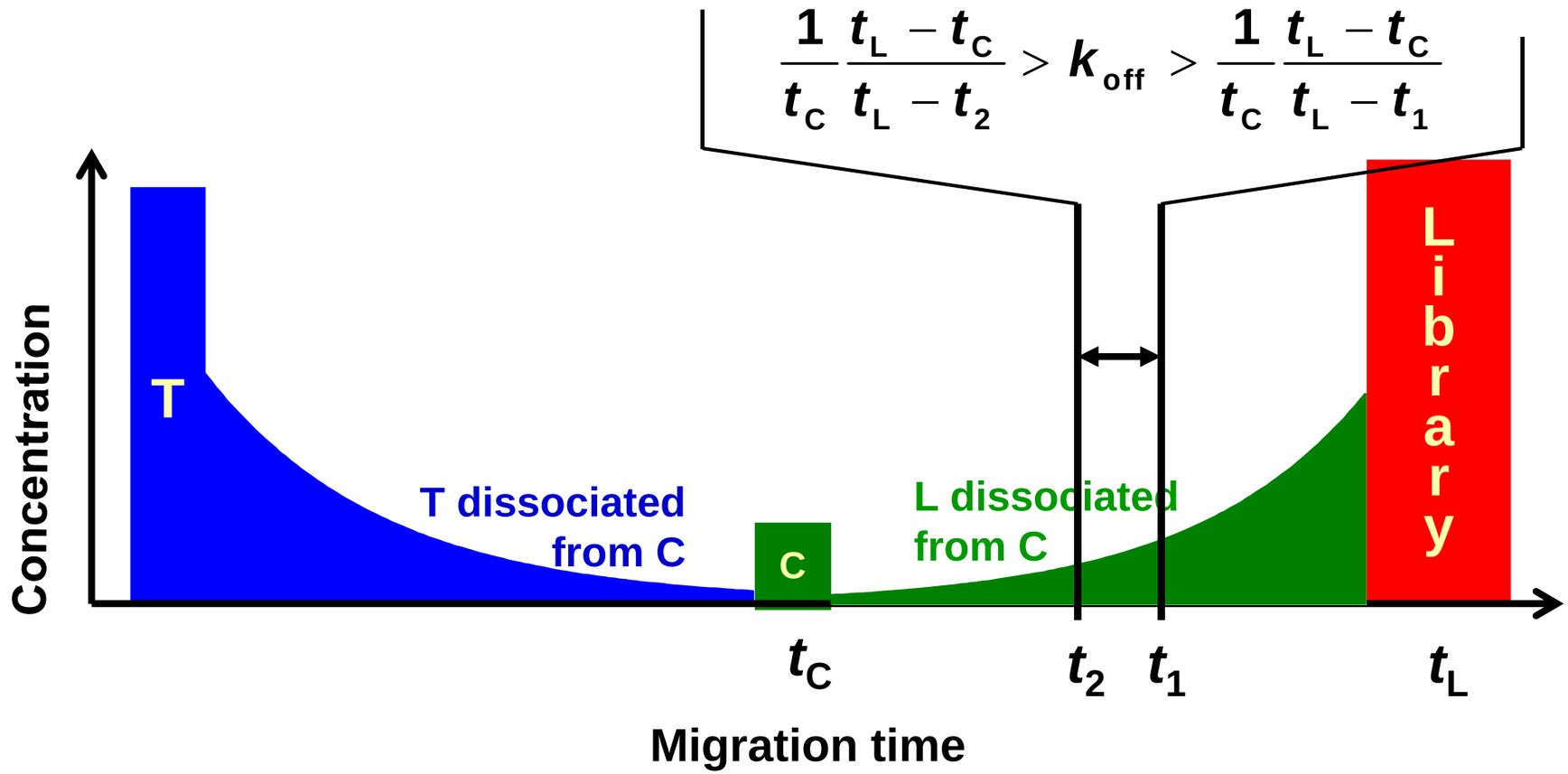
EM = Combinatorial Library of ligands + Target (T)





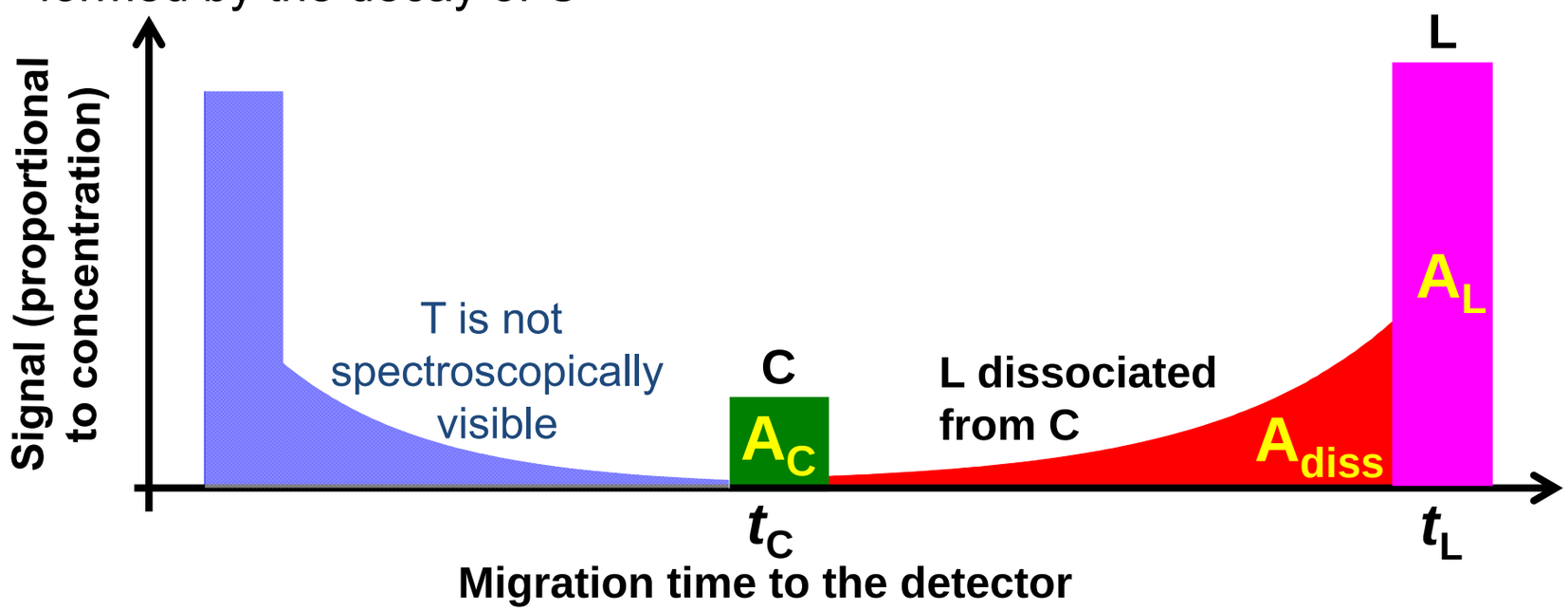


Concept of Kinetic-Separation-based selection of smart ligands with desirable k_{off}



Concept of Kinetic-Separation-based
(i) determination of k_{off} and k_{on} and
(ii) calibration-free quantitative analysis of T

Case 1: the 3 zones are separated ⇒ we can assume that there is no rebinding of T and L formed by the decay of C

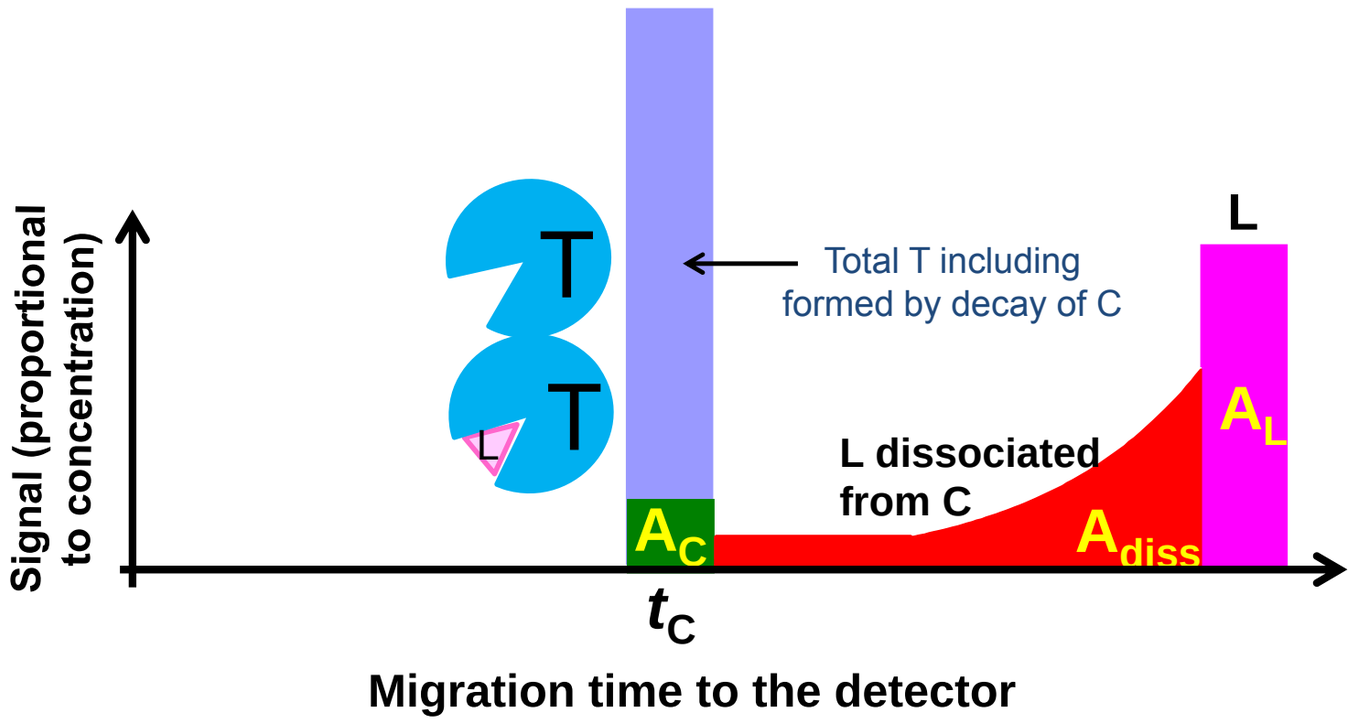


$$1)\quad k_{off} = \ln\left(\frac{A_C + A_{diss}}{A_C}\right) / t_C, \qquad K_d = \frac{[T]_0\left(1 + A_L / (A_C + A_{diss})\right) - [L]_0}{1 + (A_C + A_{diss}) / A_L}, \qquad k_{on} = k_{off} / K_d$$

$$2)\quad [T]_0 = K_d \frac{A_{diss} + A_C}{A_L} + [L]_0 \frac{1}{1 + A_L / (A_{diss} + A_C)}$$

Concept of Kinetic Separation-based
(i) determination of k_{off} and k_{on} and
(ii) calibration-free quantitative analysis of T

Case 2: T is not separated from C ⇒ the presence of T in the zone of C results in re-binding of L (formed from the decay of C) to the excess of T

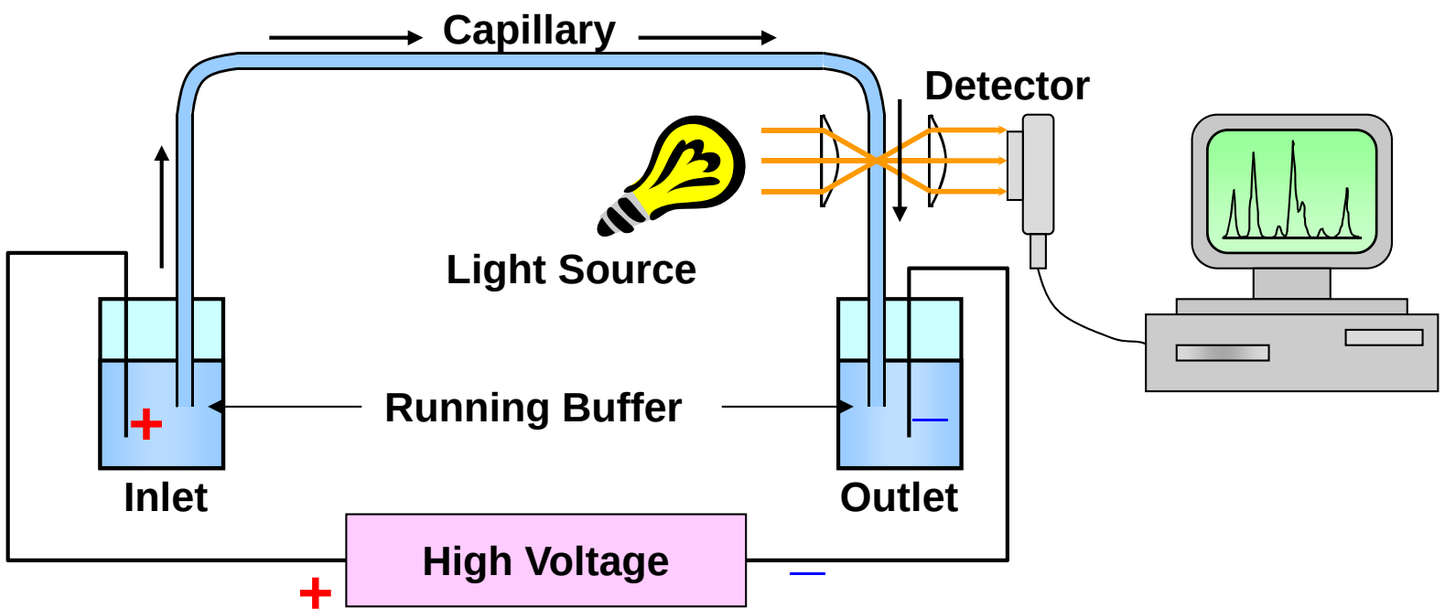


- No simplifying assumptions can be made for finding k_{on} and k_{off} or $[T]_0$
- Fitting an experimental concentration profile by a simulated one is required to find k_{on} and k_{off} or find $[T]_0$ for known k_{on} and k_{off}

Part 3

Examples of applications of Kinetic Capillary Electrophoresis (KCE) with optical detection

Schematic of a Capillary Electrophoresis (CE) instrument

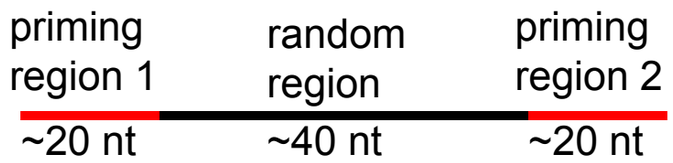


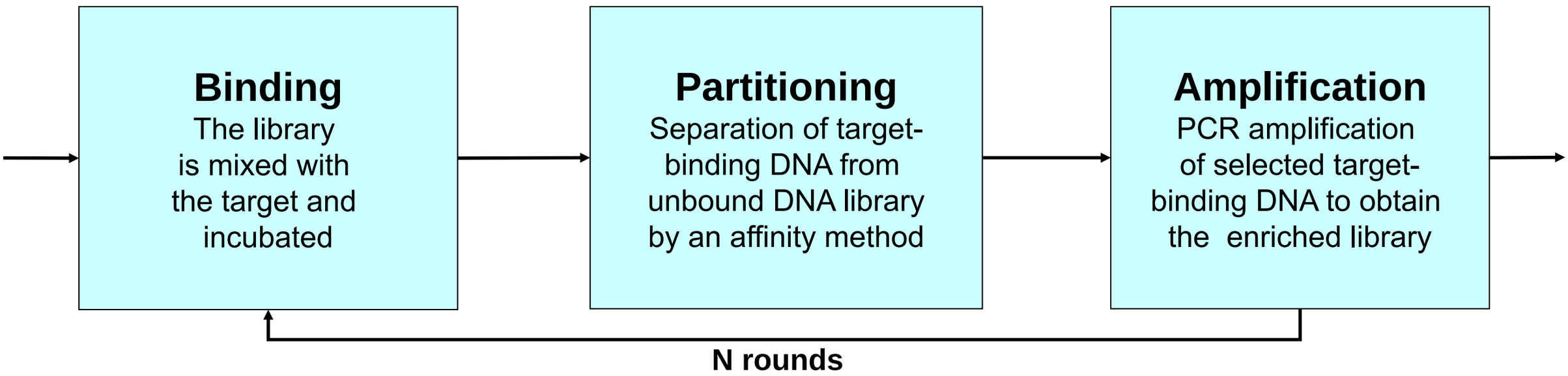
Application 1

Selection of DNA aptamers by KCE

SELEX

Selection of binding ligands from a ssDNA library with starting sequence diversity of 10^{12}-10^{15}

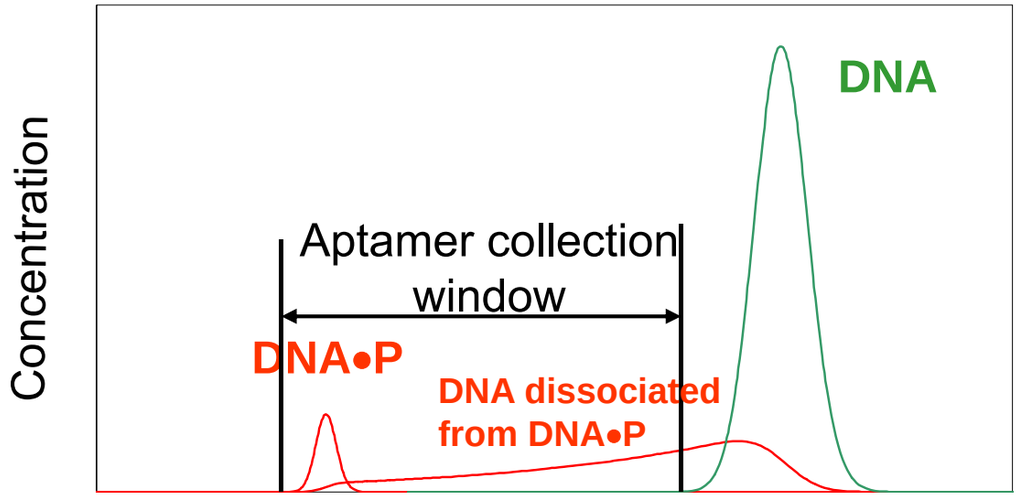




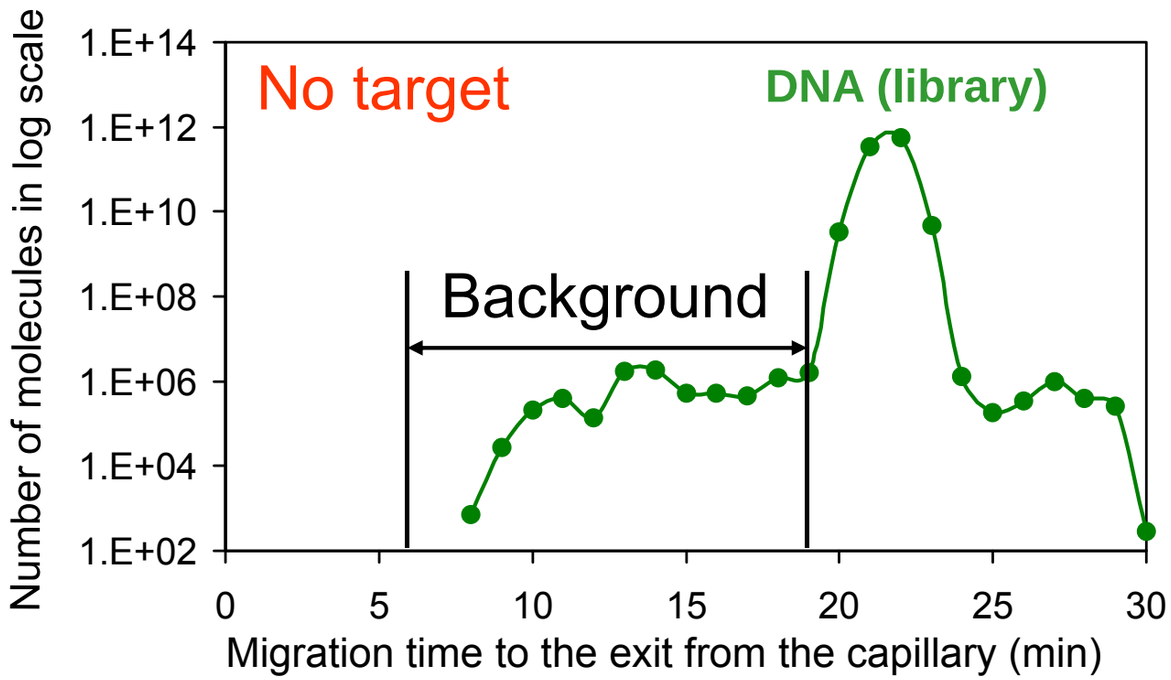
- Conventional SELEX uses partitioning on surfaces, *e.g.* on filters
- Background of partitioning is typically 1-10%
- Required number of rounds of selection is typically more than 10
- Smart aptamers (e.g. with pre-defined k_{off} or K_d) are hard to obtain

Advantage of KCE-based partitioning is exceptionally low background

Schematic



Experiment



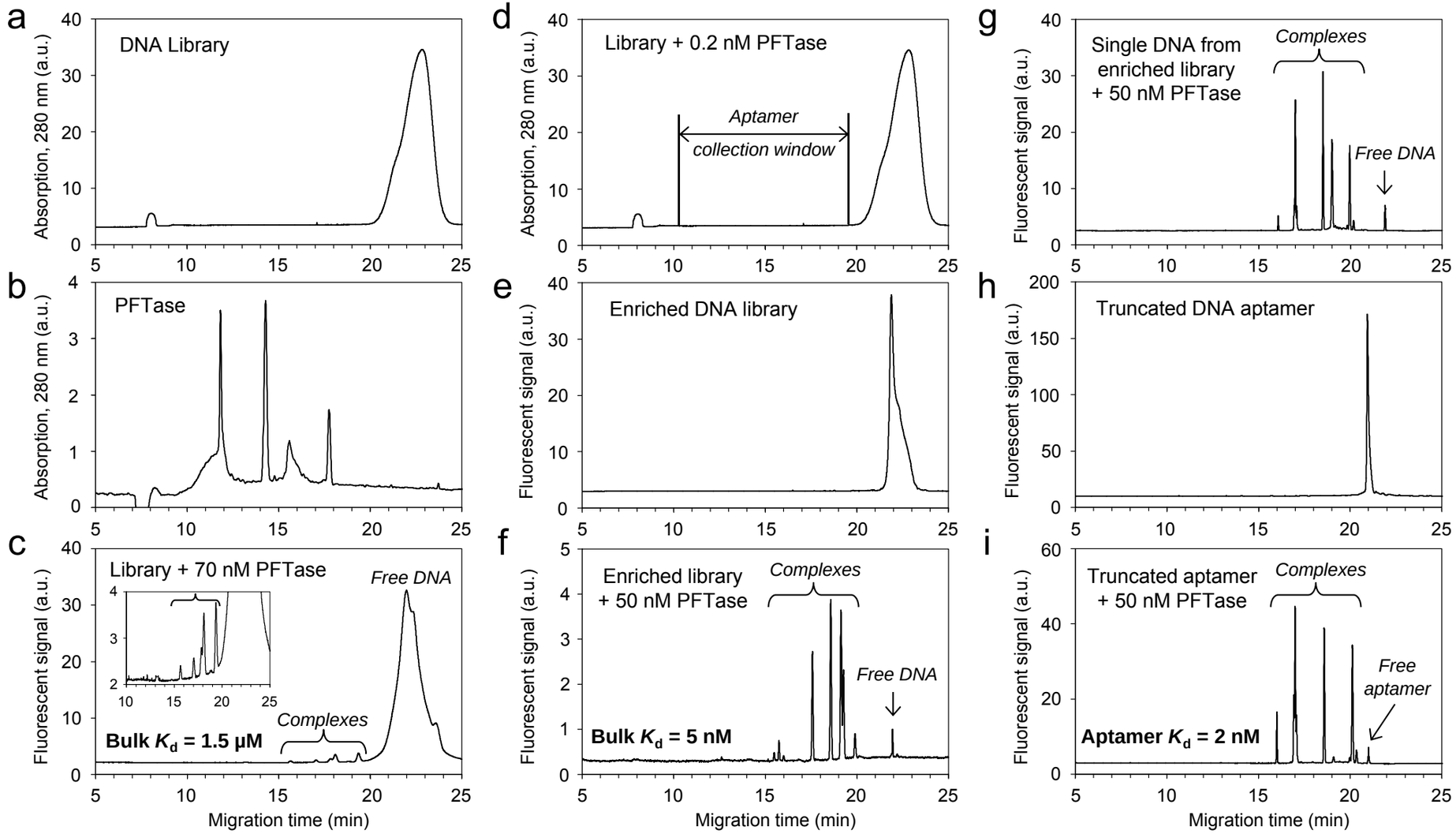
Background is the relative amount of DNA in the aptamer collection window without target protein

For KCE: Background < 0.001%

For other methods: Background > 0.1%

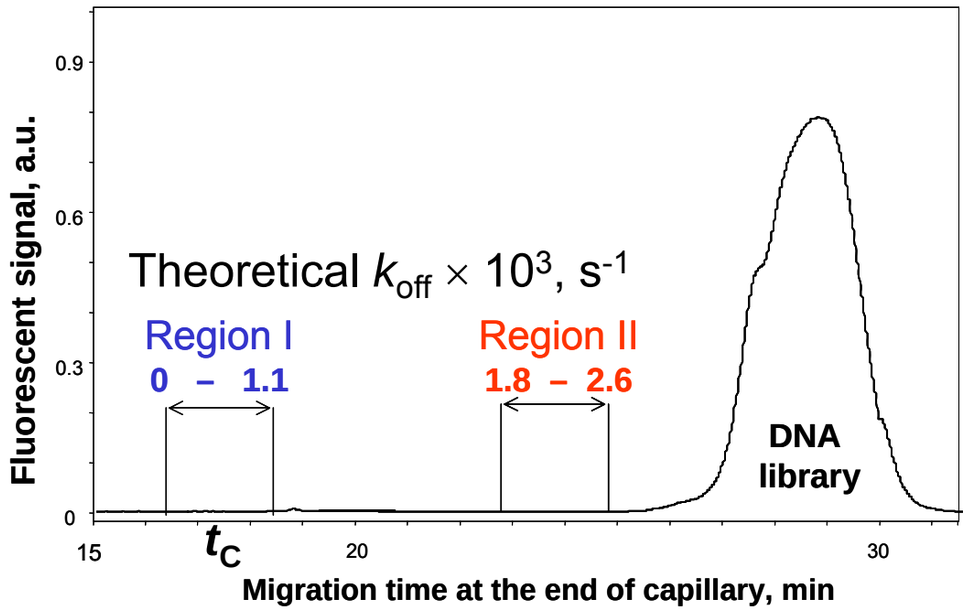
Example 1:

One-step selection of naive aptamers to farnesyltransferase



Example 2:

Selection of smart aptamers with pre-defined k_{off} for MutS protein



	k_{off} (theoretical), $x10^{-3}$ s^{-1}	k_{off} (experimental), $x10^{-3}$ s^{-1}	K_d (experimental), nM
Region I	0 – 1.05	**0.4**	**11**
Region II	1.76 – 2.64	**1.7**	**44**

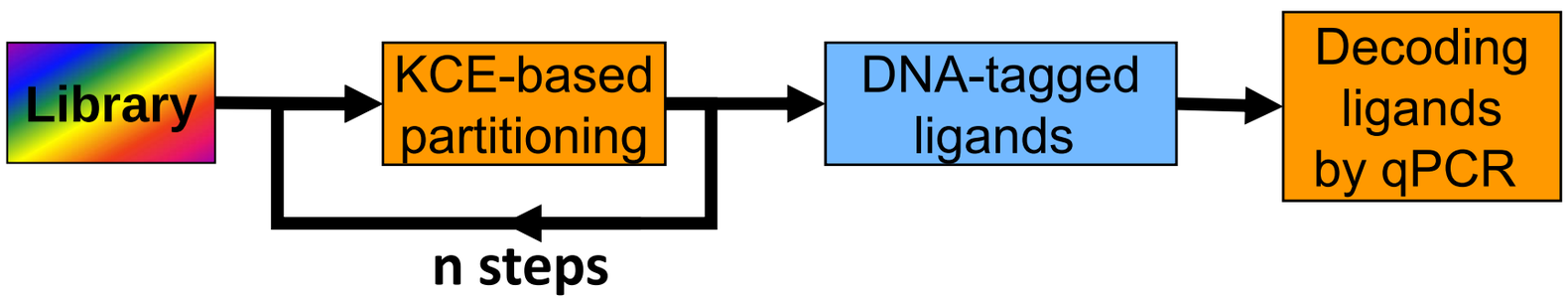
Application 2

KCE-based selection of drug leads from libraries of DNA-encoded small molecules

(current work in collaboration with GlaxoSmithKline)

High efficiency of KCE-based partitioning is attractive for selection of binding ligands from libraries of DNA-encoded small molecules

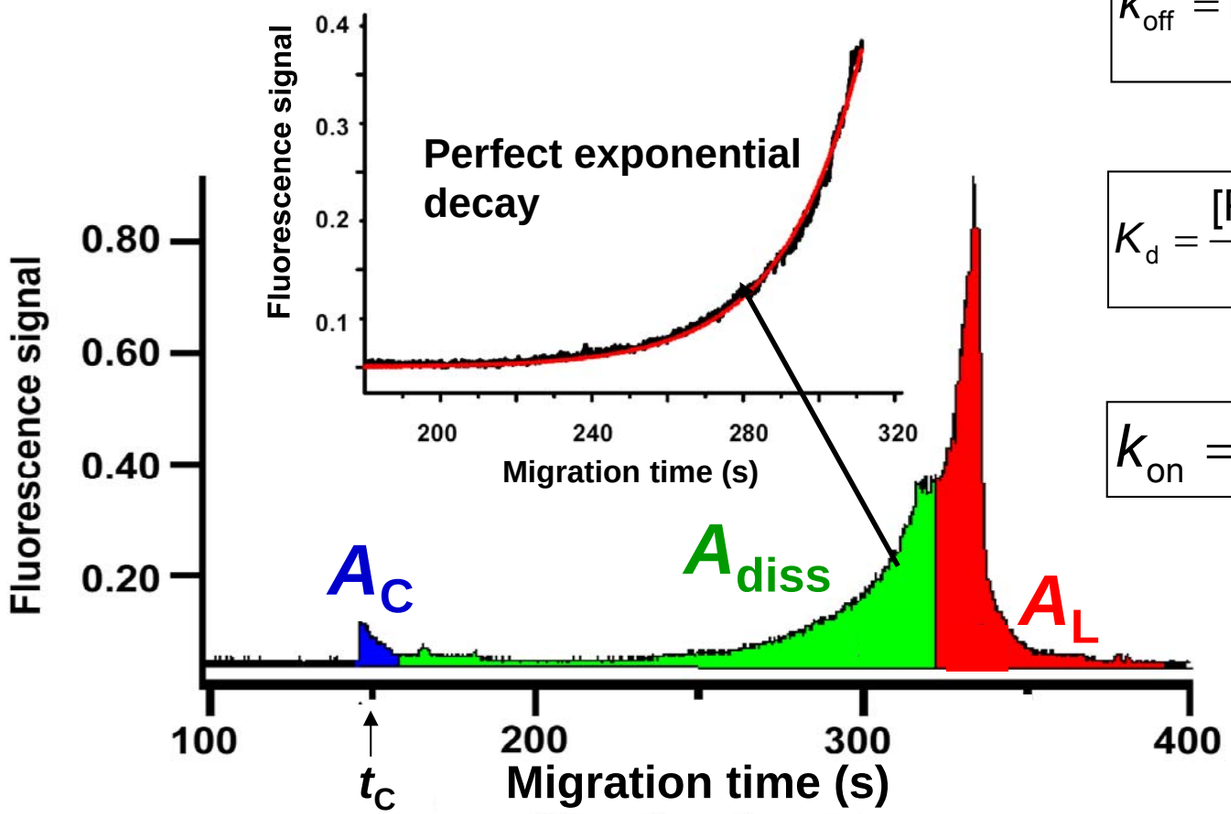
Structural Diversity > 10^9



Application 3
KCE-based determination of and k_{off} and k_{on} of protein-DNA binding

Ligand is fluorescently labeled DNA
Target is unlabeled SSB protein

→ Free protein is undetectable



$$k_{off} = \ln\left(\frac{A_C + A_{diss}}{A_C}\right)/t_C \approx 3\times10^{-2}\,s^{-1}$$

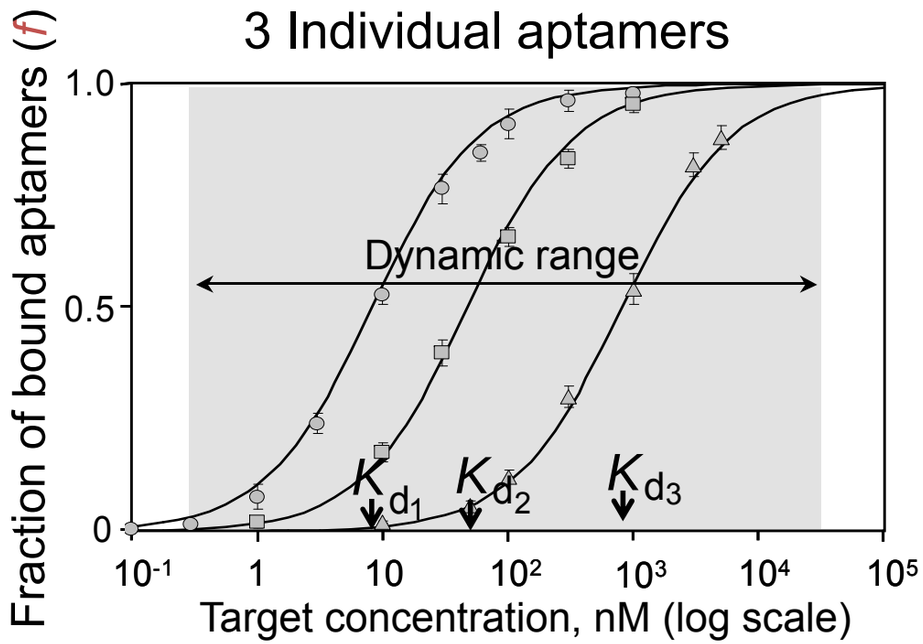
$$K_d = \frac{[P]_0\left(1+A_L/(A_C+A_{diss})\right)-[L]_0}{1+(A_C+A_{diss})/A_L} \approx 2.8\times10^{-7}\,M$$

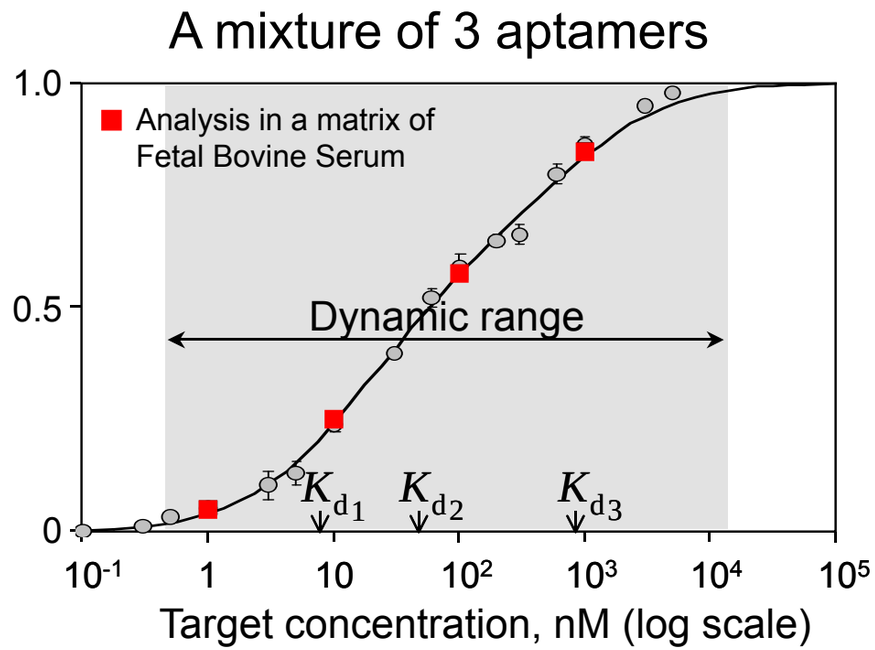
$$k_{on} = k_{off}/K_d = 1.2\times10^{5}\ M^{-1}s^{-1}$$

Application 4

KCE-based calibration-free analysis of MutS protein with ultra-wide dynamic range

3 Smart Aptamers with K_d of 7.6, 46, and 810 nM were used





$[T]_0$ is found by solving the following algebraic equation for *n* aptamers (L):

$$\sum_{i=1}^{n} \frac{[L]_{0_i}}{K_{d_i} + [T]_0 - f \times \sum_{j=1}^{n} [L]_{0_j}} = \frac{f \times \sum_{i=1}^{n} [L]_{0_i}}{[T]_0 - f \times \sum_{i=1}^{n} [L]_{0_i}}$$

Part 4

Examples of applications of Kinetic Size-Exclusion Chromatography (KSEC) with MS detection

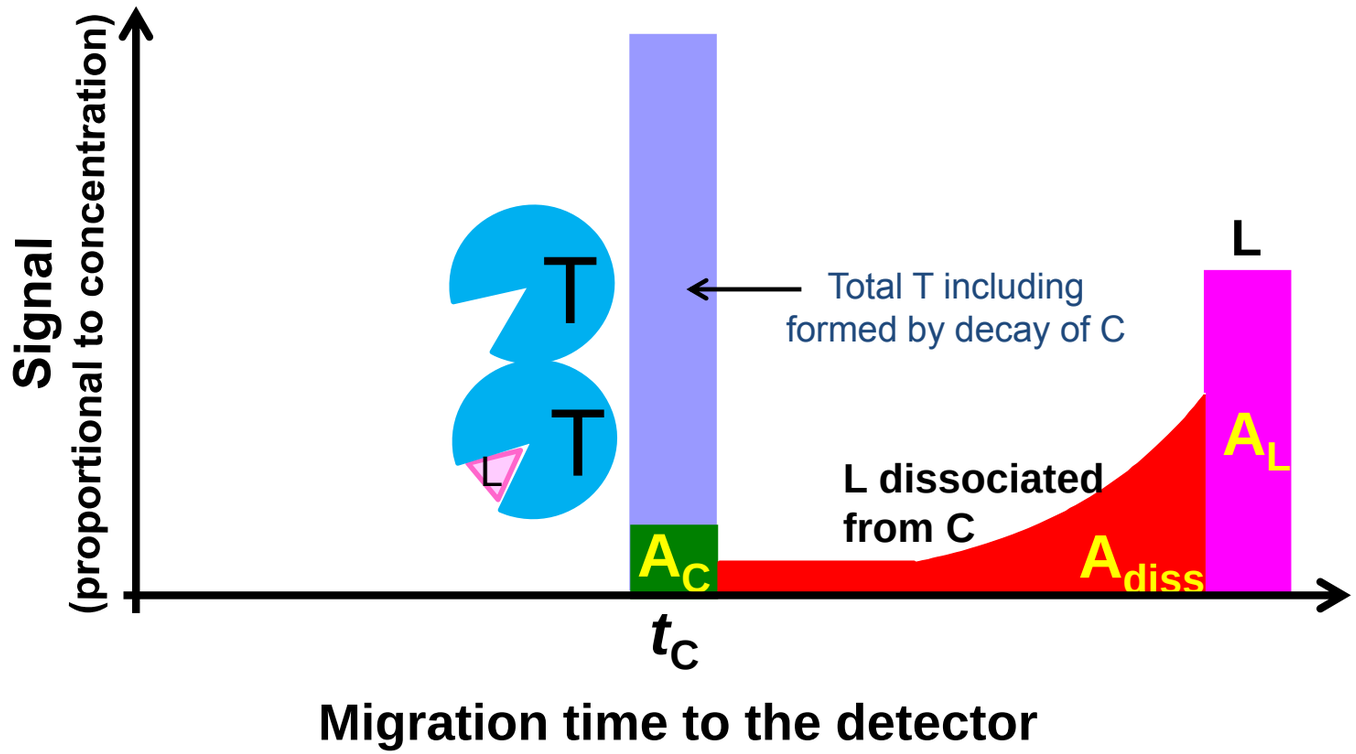
Instrumentation for KSEC-MS

Separation of L from C on a SEC column by HPLC

MS detection of signal proportional to [L]

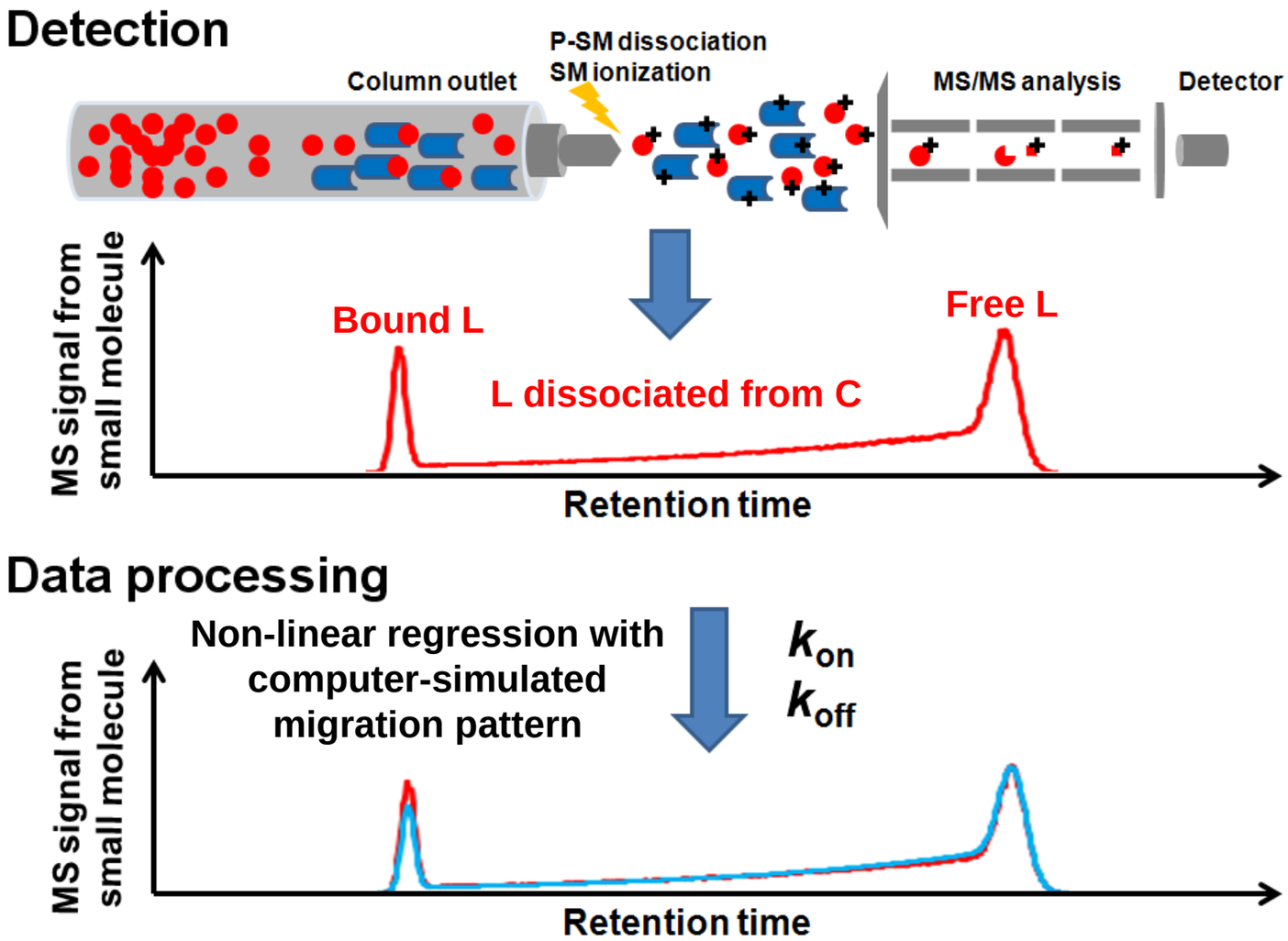
Application 1

Determination of k_{on} and k_{off} for binding of a protein to a small-molecule drug



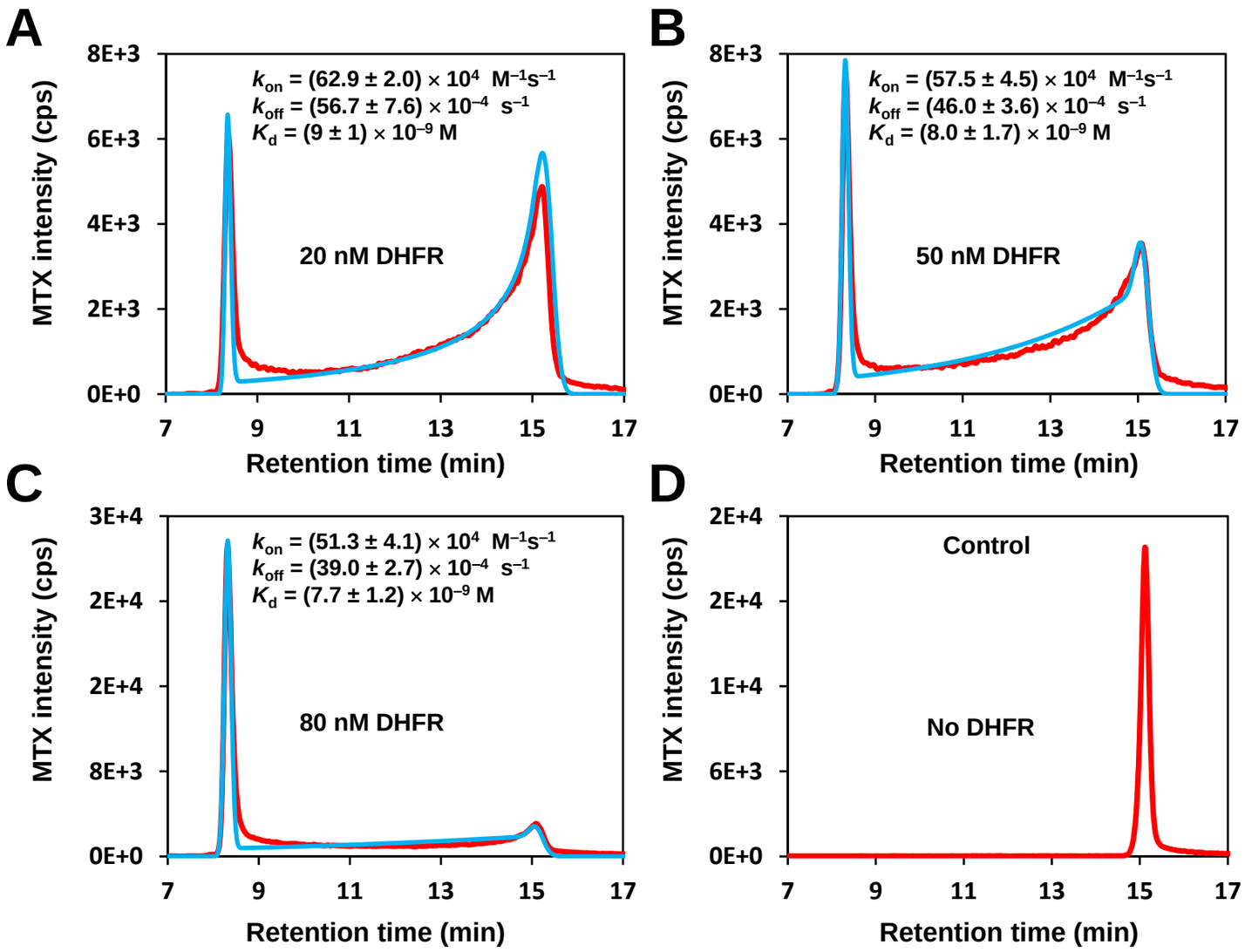
Fitting an experimental signal from L by a simulated one is required to find k_{on} and k_{off}

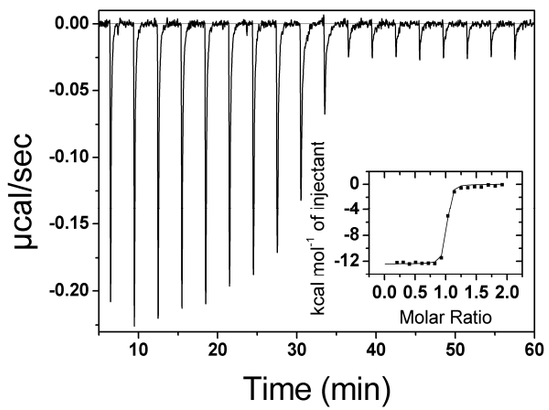
Small molecule detection and determination of k_{on} and k_{off}



Example 1:

Interaction between dihydrofolate reductase (DHFR) and Methothrixate (MTX)





KSEC-MS global fitting:

k_{on} = (60.8 ± 6.3) × 10⁴ M⁻¹s⁻¹

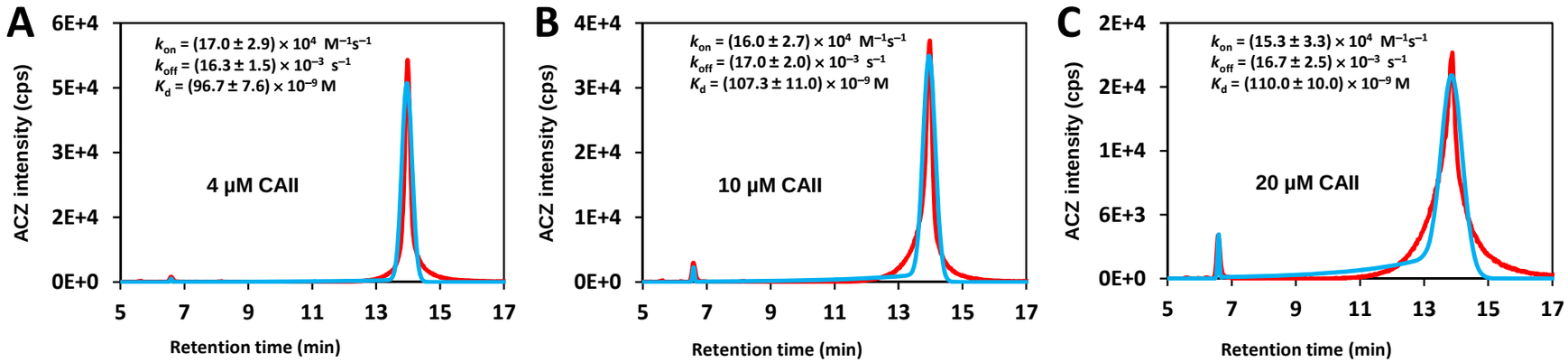
k_{off} = (5.6 ± 1.3) × 10⁻³ s⁻¹

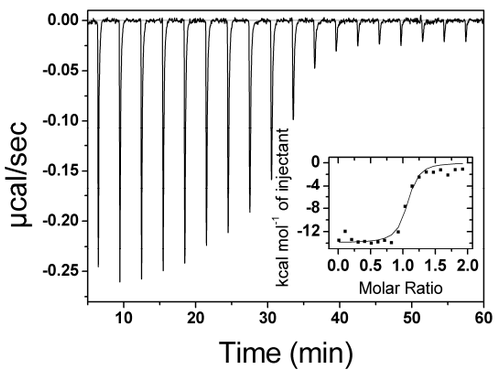
K_d = (9.1 ± 1.4) × 10⁻⁹ M

ITC: K_d = (10.2 ± 0.8) × 10⁻⁹ M

Example 2

Interaction between carbonic anhydrase (CA) and acetazolamide (ACZ)





KSEC global fitting

k_{on} = (15.4± 2.2) × 10^4 $M^{-1}s^{-1}$

k_{off} = (17.8 ± 2.0) × 10^{-3} s^{-1}

K_d = (116.9 ± 15.6) × 10^{-9} M

ITC: K_d = (76.4 ± 5.3) × 10^{-9} M

Conclusions

Kinetic Separation is an “Analytical Swiss Army Knife”

Science is always wrong. It never solves a problem without creating ten more!

George Bernard Shaw

More problems

1. Interfacing CE with MS for **Kinetic Separation**
2. Development of **Kinetic Separation** tools for studying protein-protein binding
3. Development of **Kinetic Separation** tools for studying binding stoichiometry
4. Development of **Kinetic Separation** for studying kinetics of assembly/disassembly of complex molecular machines
5. Finding solutions for **Kinetic Separation** with physiological run buffers
6. Expanding **Kinetic Separation** to new other separation modes (*e.g.* ultracentrifugation)

Krylov's Team