Lesson 2

- Misura delle costanti di binding
- Metodi analitici
- NMR
- Fluorescenza
- UV vis
- calorimetria

Measurement of binding constants

$$H + G \underset{}{\overset{K}{\rightleftharpoons}} H \bullet G$$

in reality

$$\left(H \bullet S + G \bullet S \rightleftharpoons (H \bullet G) \bullet S + S \bullet S \right)$$

explicit solvent is not used because ΔG° for the association constant
reflects the stability of solvated H and G relative to solvated H•G and released solvent

$$K_a = \frac{[H \bullet G]}{[H][G]} \quad (M^{-1}) \qquad K_d = \frac{[H][G]}{[H \bullet G]} \quad (M)$$

$$\Delta G^\circ = -RT\ln(K_a)$$

assuming activity = concentra\tion

Binding constant

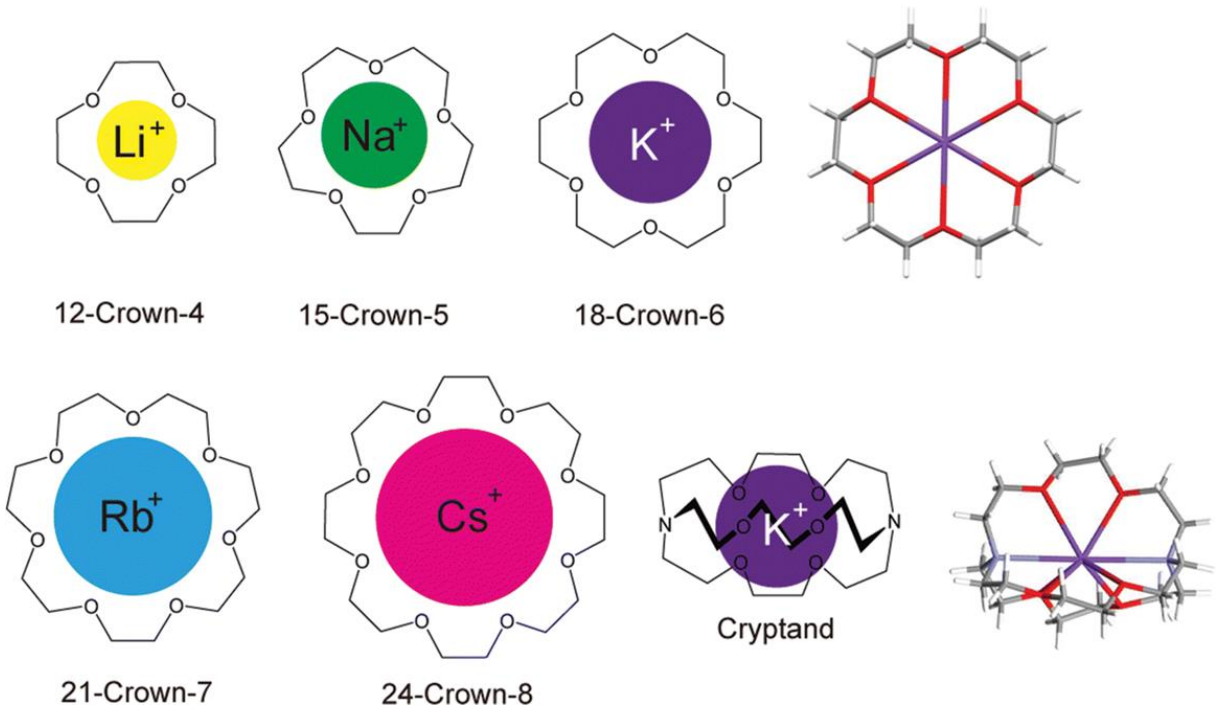
- The thermodynamic stability of a host/guest complex is measured by the binding constant. Dimensionless, but M^{-1} for 1:1 complex
- Binding constant: association or formation constant: $K_b = K_a = K_f$
- For biological processes (and drug/target interaction), dissociation constant is measured (Kd)
- The Kd is useful because is the direct measure of the concentration below which a complex dissociates
- Binding constant for a metal cation guest and a macrocyclic host, in water:

$$M(H_2O)n^{m+} + L \overset{Ka}{\rightleftharpoons} ML^{m+} + nH_2O$$

$$[ML^{m+}]/[M(H_2O)_n{}^{m+}].[L]$$

Order of magnitudo of K_b

- Kb of crown ethers for alkali metals in water 10^1-10^2
- In methanol 18-crown-6 for K^+ 10^6
- Cryptand[2.2.2] for K^+ 10^{10}



Measuring binding constants

In principle, binding constants may be assessed by any experimental technique that can yield information about the concentration of a complex, [Host·Guest], as a function of changing concentration of the host or guest.

In practice the following methods are in common use: NMR, UV, fluorescence, ITC.

In every case a concentration range must be chosen such that there is an equilibrium between significant amounts of bound and free host and guest, limiting the range of binding constants that can be measured by a particular technique.

If binding by the target host is too strong then a competing host is sometimes added in order to reduce the apparent (measured) binding constant according to the difference in guest affinity between the two hosts. T

he true affinity can then be extrapolated from a knowledge of the binding constant of the guest for the host with the lower affinity

1:1 and 1:2 host/guest complexes

- If a sequential process involves the binding of more than 1 metal cation to a host, then 2 binding constants can be measured, K11 and K12

$$M(H_2O)n^{m+} + L \rightleftharpoons ML^{m+} + nH_2O$$

$$M(H_2O)n^{m+} + ML^{m+} \rightleftharpoons M_2L^{(2m)+} + nH_2O$$

$$K_{12} = \frac{[M_2L^{2m+}]}{[M(H_2O)nm+].[MLm+]}$$

Overall binding constant:

$$\beta_{12} = K_{11} \times K_{12}$$

Magnitudes of K can vary widely, so they are reported as logK:

$$\log\beta_{12} = \log K_{11} + \log K_{12}$$

The supramolecular titration experiments

- By different techniques: UV-vis, NMR, fluorescence, ITC, SPR

a)

The simple 1:1 system

$$H + G \overset{K_a}{\rightleftharpoons} HG$$

b)

The general m:n system

$$mH + nG \overset{\beta_{mn}}{\rightleftharpoons} H_mG_n$$

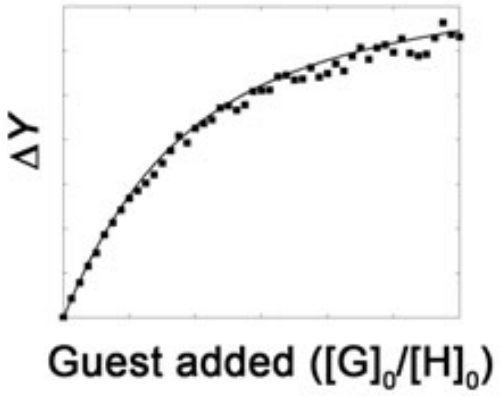
c)

The 1:2 system

$$H + G \overset{K_1}{\rightleftharpoons} HG$$

$$HG + G \overset{K_2}{\rightleftharpoons} HG_2$$

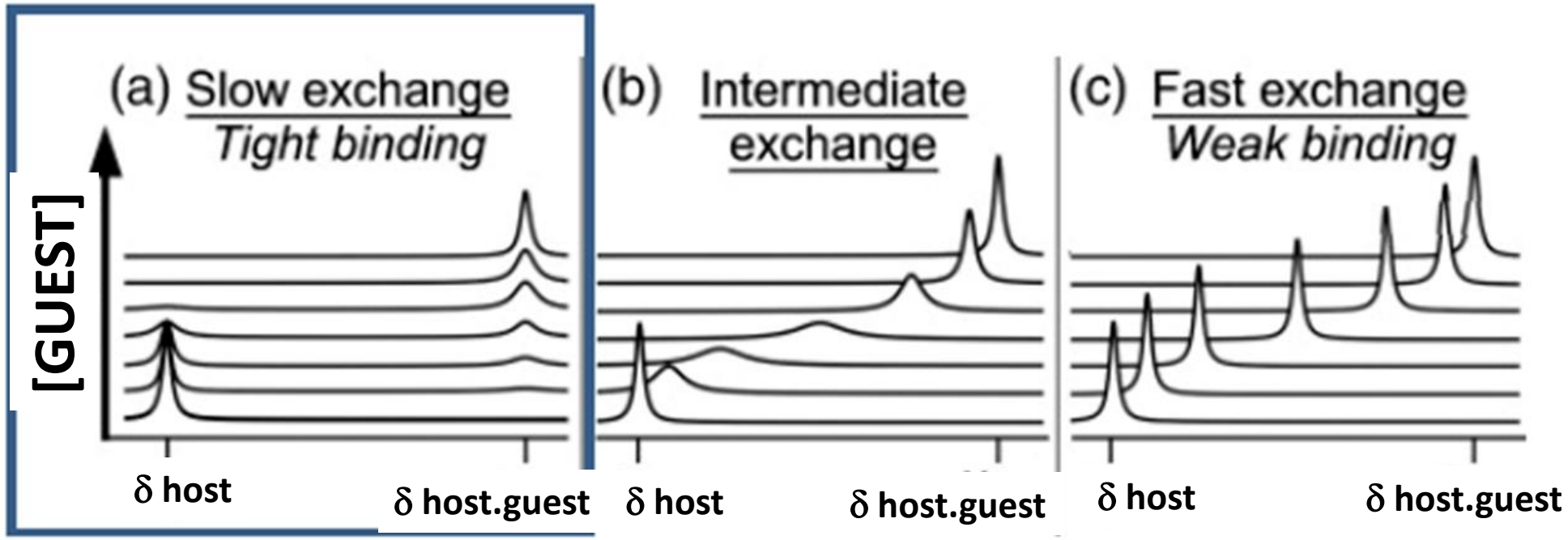
d)



(a)–(c) Three of the supramolecular equilibria : H = host, G = Guest and HxGx = host–guest complex of interest, Kx = the thermodynamic association constant for a particular
interest and bmn = overall association constant for an m : n host–guest
complex formation. (d) A typical binding isotherm.

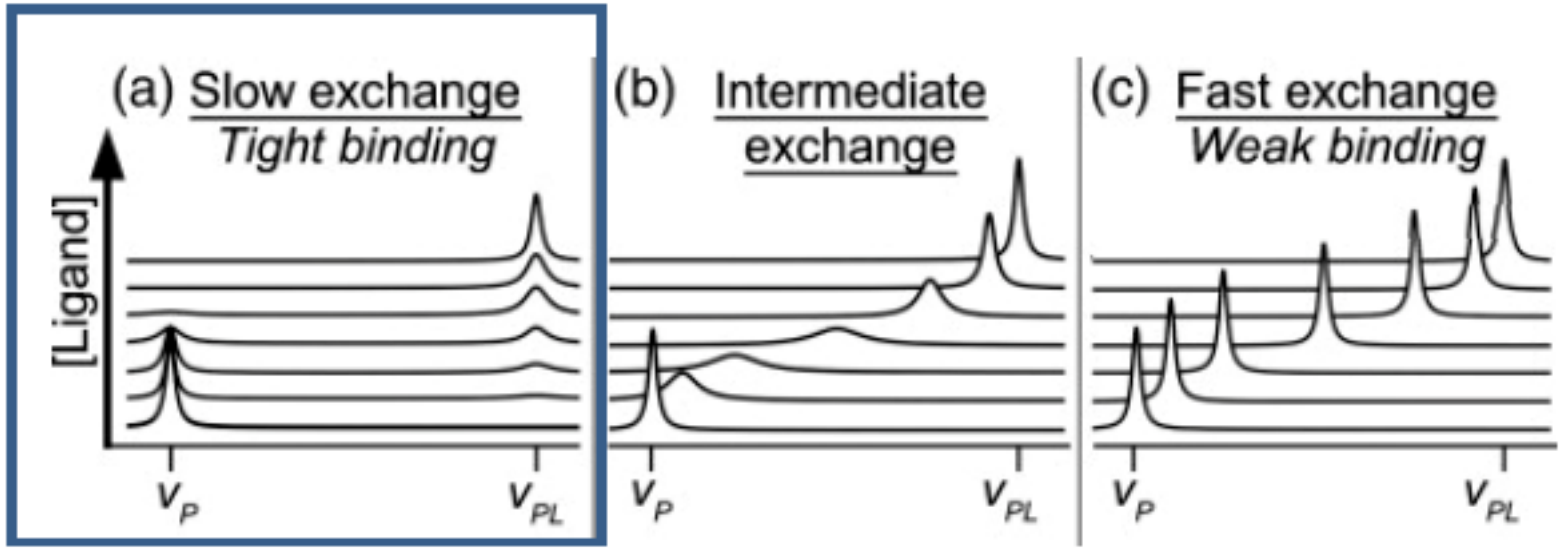
Nuclear Magnetic Resonance (NMR) titration

- NMR timescale (NMR sampling rate) vs complex formation rate



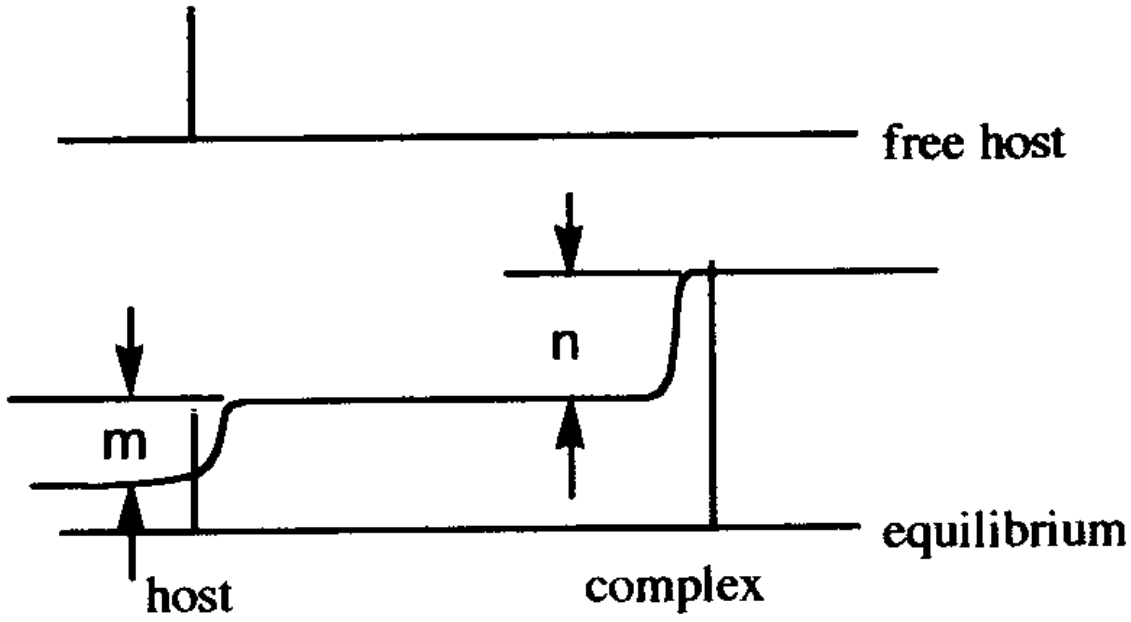
NMR titration: adding a guest to a host

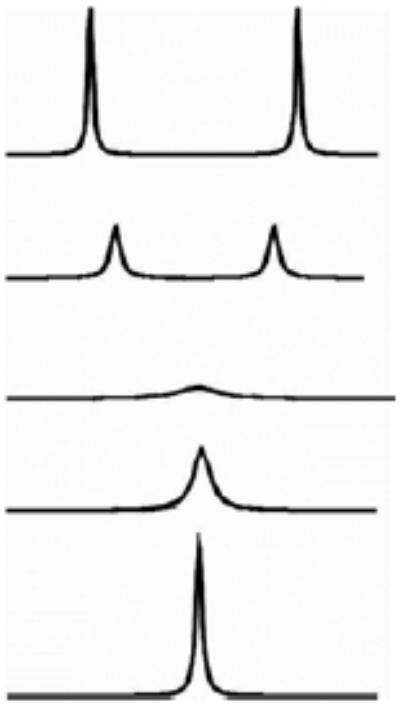
- If the exchange host-guest is slow compared to NMR timescale (high binding constant), then 2 sets of signals are visible, one for unbound and the other for the bound ligand (guest).
- Ka or Kd can be determined from simple integration of signal of bound and unbound host or guest.



Case 1: The host-guest complexation equilibrium, which has a very slow exchange rate compared to the NMR time scale

In this case the peaks which are assigned to the host parts in the complex and those to the free host are observed individually in the same NMR spectrum. Those peaks appear at individual chemical shifts.





NMR time scale
(chemical shift)

slow exchange
$k_{ex} \ll \Delta\nu$

coalescence
$k_{ex} \sim \Delta\nu$

fast exchange
$k_{ex} \gg \Delta\nu$

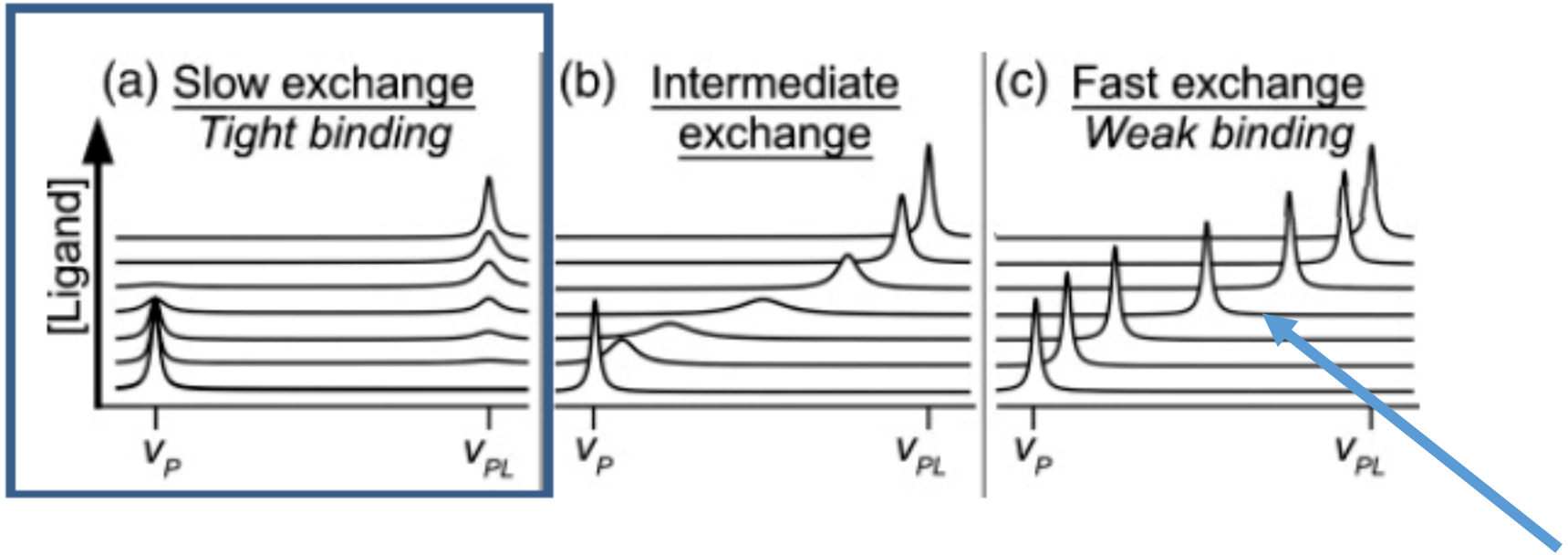
H/D exchange

amide N-^{1}H → N-^{2}D

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} B$$

$$k_{ex} = k_1 + k_{-1}$$

- Chemical or conformational exchange can be analyzed by NMR
- Rate constants can be determined, e.g. for a 2-state binding equilibrium, chemical reaction, or conformational exchange



Most host–guest equilibria are fast on the (relatively slow) NMR spectroscopic time scale, however, and the chemical shift observed for a particular resonance (that is sensitive to the complexation reaction) is a weighted average between the chemical shift of the free and bound species.

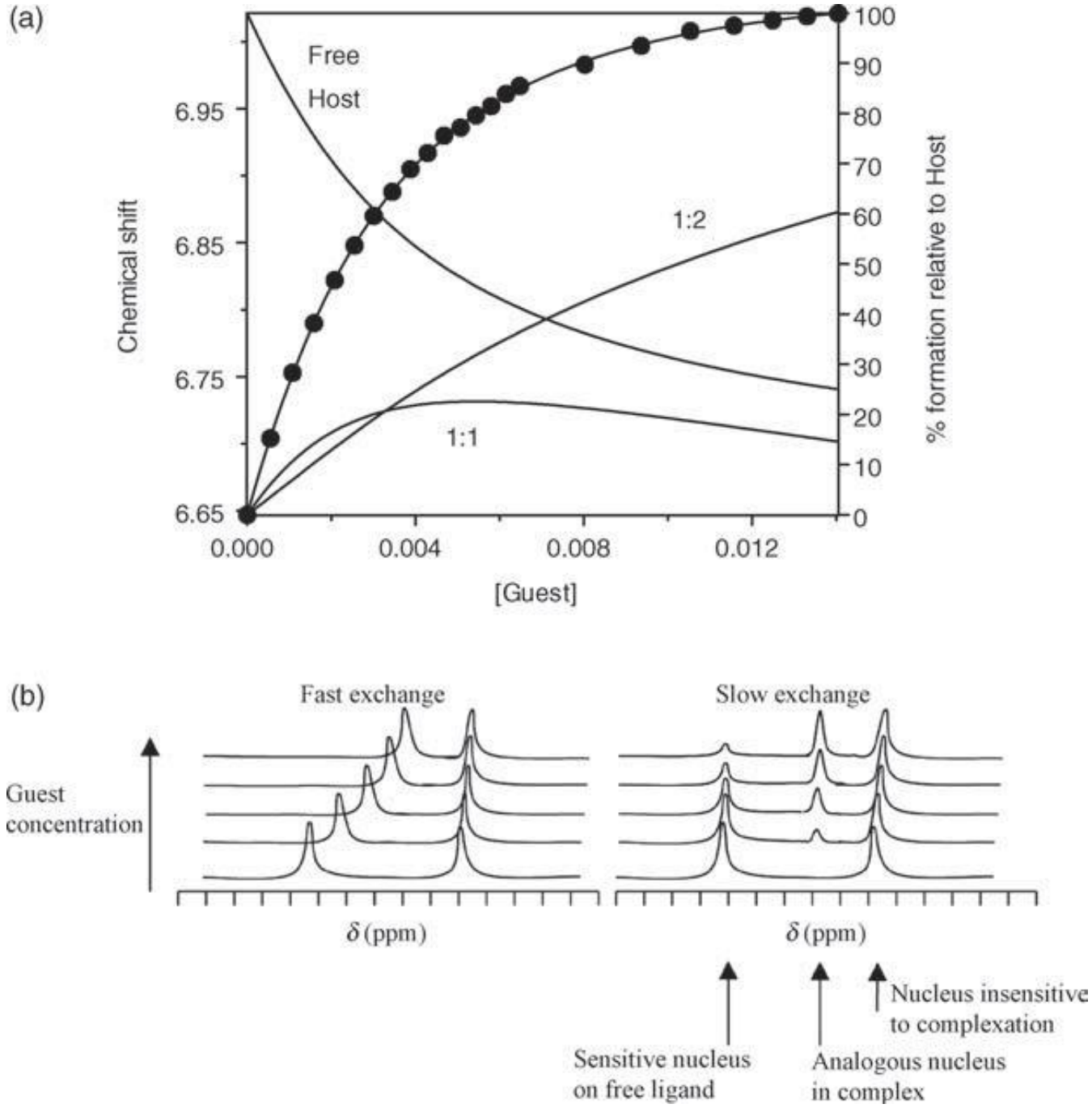
The NMR titration, information on regioselectivity and thermodynamic of binding

Commonly, changes in chemical shift ($\Delta\delta$) are noted for

various atomic nuclei present (*e.g.* 1H in 1H NMR) as a function of the influence the guest binding has on their magnetic environment. As a result, two kinds of information are gained.

Firstly, the location of the nuclei most affected may give qualitative information about the regioselectivity of guest binding (is the guest inside the host cavity?).

More importantly, however, the shape of the titration curve (a plot of $\Delta\delta$ against added guest concentration, *e.g.* Figure 1.4) gives quantitative information about the binding constant.



(a) NMR titration plot (isotherm) and corresponding speciation plots for a system undergoing fast equilibration on the NMR time scale, with log β11 = 2.3 and log β12 = 4.5.

(b) Schematic NMR spectra of slowly equilibrating mixtures of free host, guest and host–guest complex.

NMR spectroscopic methods are useful for binding constants in the range $10–10^4$ M-1.

Such titration curves are often analysed by computer least-squares curve fitting (e.g. by a program such as EQNMR6) using Equation 1.14 to determine optimum values of δmn (chemical shift of each species present where mn is the ratio of host, H, and guest, G) and βmn (stepwise binding constant).

The isotherm shown in Figure 1.4a fits a stoichiometry model involving both 1:1 and 1:2 host:guest complexes with log β11 = 2.3 and log β12 = 4.5. The plot also shows the relative percentage amounts of each species present in the solution for a given host and guest concentration.

a)

The simple 1:1 system

$$H + G \overset{K_a}{\rightleftharpoons} HG$$

b)

The general m:n system

$$mH + nG \overset{\beta_{mn}}{\rightleftharpoons} H_mG_n$$

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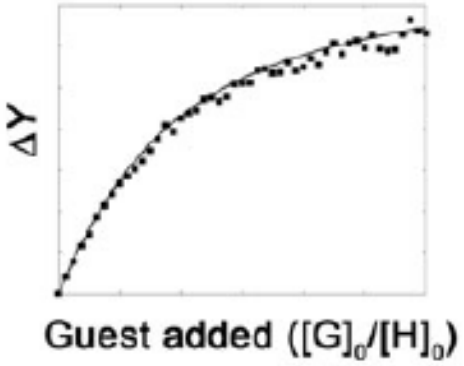
d)



- In a typical NMR titration experiment small quantities of guest are added to a solution of the host at known concentration in deuterated solvent.
- The NMR is monitored in function of [G] or H:G ratio.
- Normally changes in chemical shifts ($\Delta\delta$) of several nuclei (^{1}H in ^{1}H-NMR) are noted as a consequence of the influence of host binding in the guest chemical environment.

Method of Continuous Variation (Job Plots)

A key aspect of such calculations is the use of the correct stoichiometry model (i.e. the ratio of host to guest, which must be assumed or determined).

There is a strong bias in the supramolecular chemistry literature towards the fitting of data to 1:1 stoichiometries, and it is a common mistake to neglect higher aggregates.

Binding stoichiometry may be confirmed in most kinds of titration experiments that allow the concentration of complex to be determined by making up a series of solutions with varying host:guest ratios such that the total concentration of host and guest is a constant.

Method of continuous variations (Job Plot)

- In the job plot the [H.G] against the [H]/[H] + [G] (molar fraction of H) is plotted.
- In the vertical axis the $\Delta\delta$ vs molar fraction in horizontal axis is plotted
- For 1:1 complex a bell-shaped curve is obtained with a maximum at 0.5
- For 2:1 complex the maximum is located at 0.66

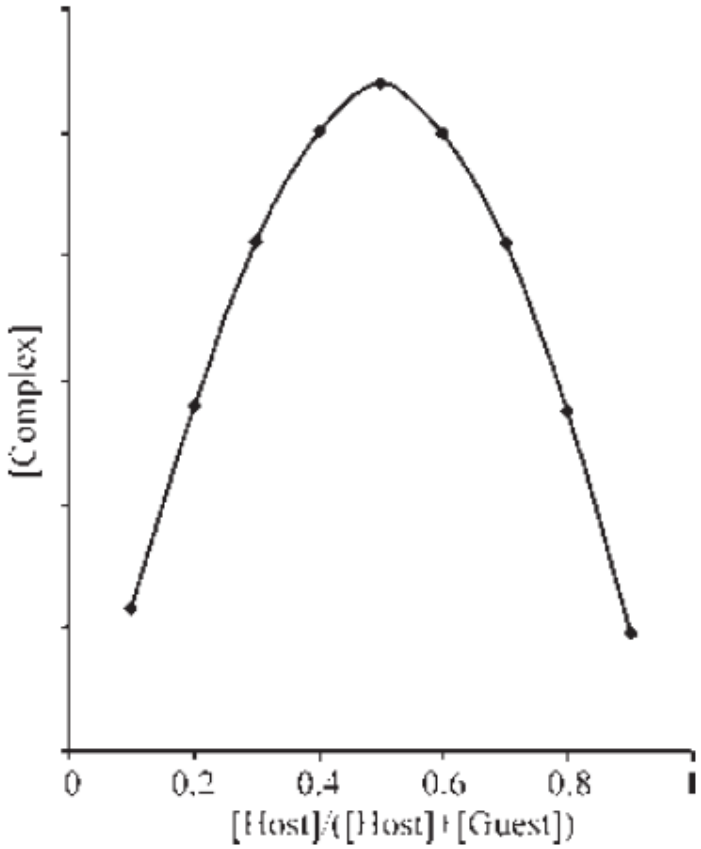
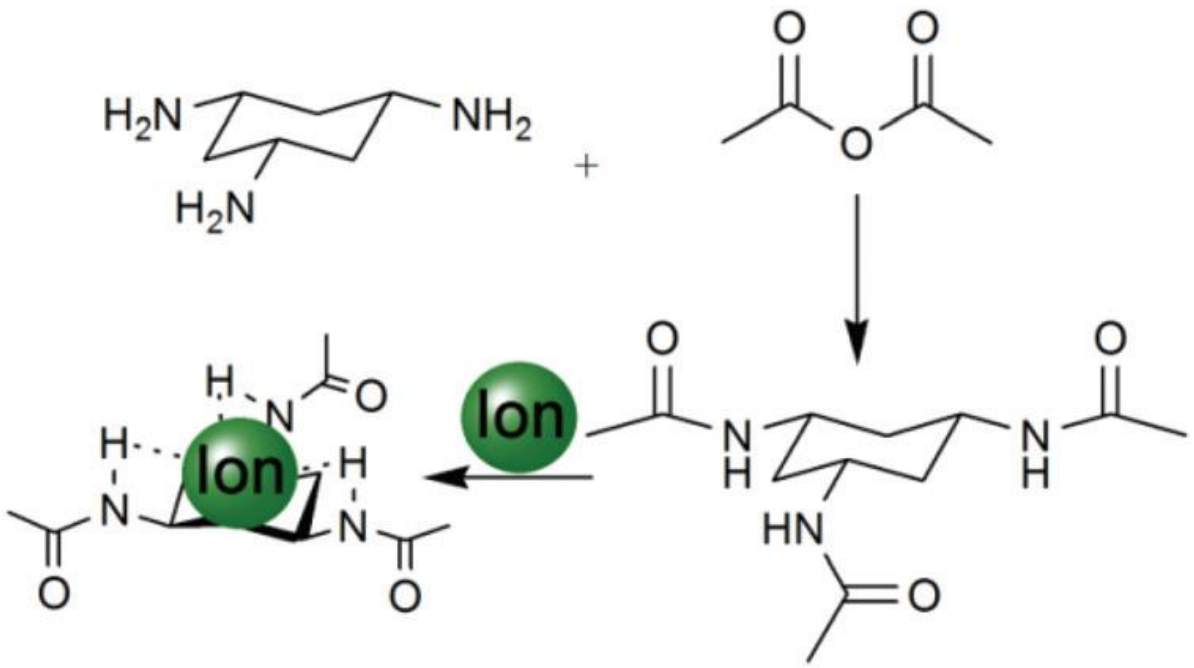
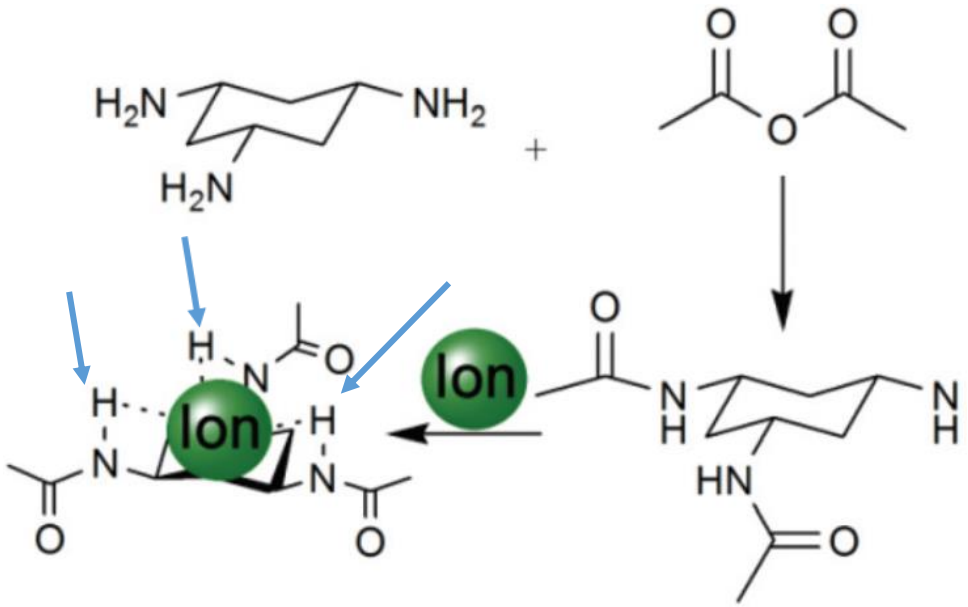


Figure 1.5 Job plot for a 1:1 host–guest complex.

Example (Inorg. Chem. Front., 2014,1, 49)



- Host: cis,cis-1,3,5-triaminocyclohexane (cis-tach)
- Guest: anione Cl^-, Br^-, I^-



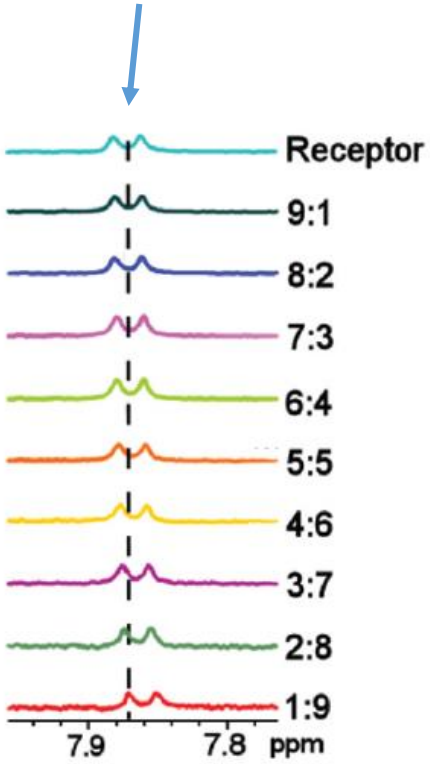


Fig. 3 ^{1}H NMR (d_6-DMSO) showing the shift of the amide NH when hydrogen bonding to chloride occurs; ratio is receptor to anion.

^{1}H NMR experiments were carried out for each of the mixtures and the receptor where a plot of Δδ (where Δδ = the shift of the receptor – (shift of the receptor + guest)) multiplied by the mole fraction was then plotted against mole fraction.

It was observed that the NH chemical shifts of the receptor shifted upfield in the presence of the anionic guest, indicating that complexation had occurred.

2 informations:

- 1) the **location of nuclei that are shifted** gives qualitative informations on the regiochemistry of binding
- 2) the **shape of binding curve** ($\Delta\delta$ vs [G])gives information on the Ka of binding. NMR binding experiments are useful for the determination of Ka between 10 and 10^4 M^{-1}.

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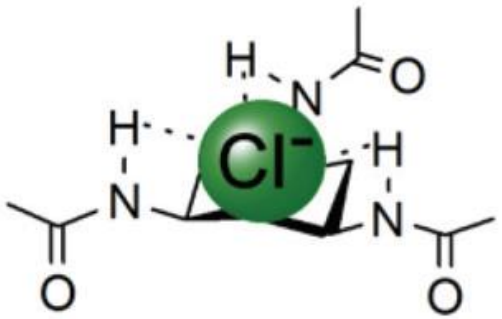
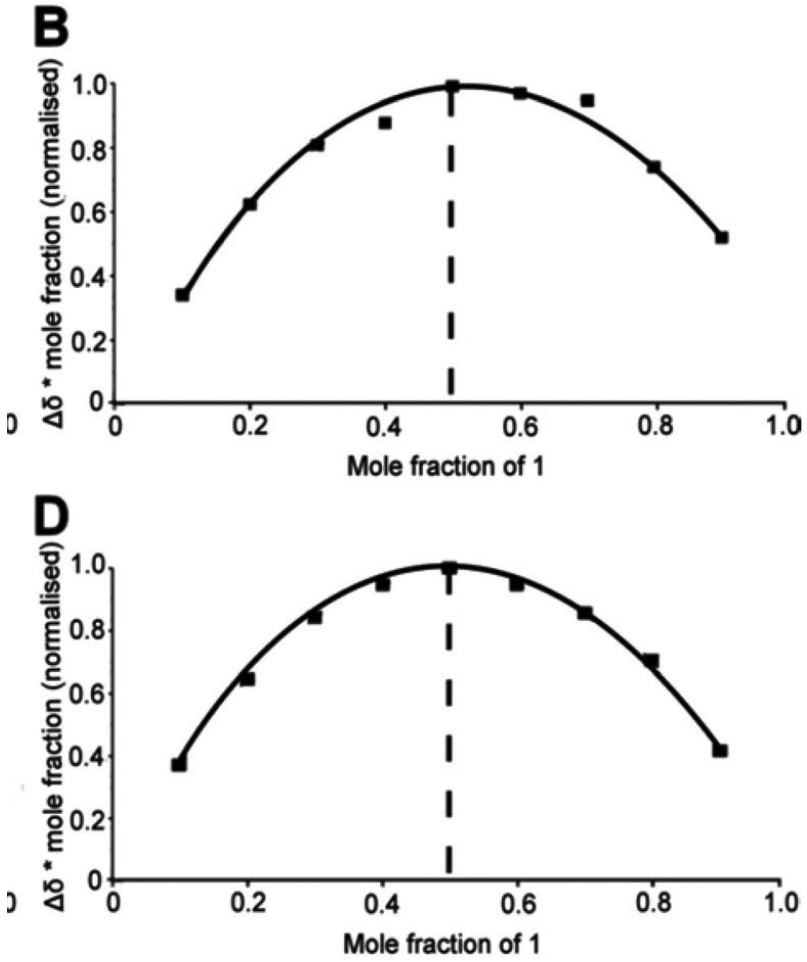
Stoichiometry of the cis-tach/anion complex





- A Job plot was then produced for each anion with the maximum at 0.5 for each, corresponding to a stoichiometry of 1 : 1

NMR titration of cyclodextrin-vanillin inclusion complex

NMR Spectroscopic characterization of β-cyclodextrin inclusion complex with vanillin

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Abstract. The inclusion of vanillin by β-cyclodextrin was investigated by ^{1}H NMR. The continuous variation technique was used to evidence the formation of soluble 1:1 complex in aqueous solution. The association constant of vanillin with β-cyclodextrin has been obtained at 298 K by fitting the experimental chemical shifts differences, $\Delta\delta_{obs} = \delta_{free} - \delta_{obs}$ of the observed guest and host protons, with a non-linear regression method. Besides the effective association constant, the fitting procedure allows a precise determination of all chemical shift parameters characterizing the pure complex. They can by used for an analysis of the geometry of the molecular complex in solution.

Cyclodextrins (CDs) are cyclic oligosaccharides containing six (α-CD), seven (β-CD) or eight (γ-CD) α-1,4-linked glucopyranose units with a hydrophilic hydroxyl group on their outer surface and a hydrophobic cavity in the center. The hydrophilic exterior of the CD molecules can make them water soluble, but the hydrophobic cavity provides an environment for appropriate sized non-polarmolecules.

In aqueous solution CDs are capable of forming inclusion complex with many molecules by taking up a whole molecule or some part or it, into the cavity. These non-covalent complexes offer a variety of physicochemical advantages over uncomplexed molecules including increased water solubility and stability.

In this paper, we report a 1H NMR study of the inclusion complex formed between vanillin (figure 1a) and β-cyclodextrin (figure 1b), in aqueous medium

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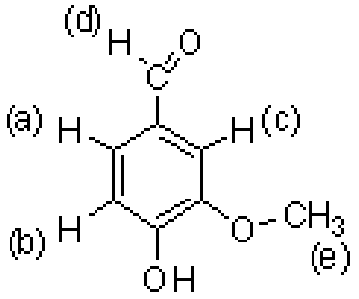
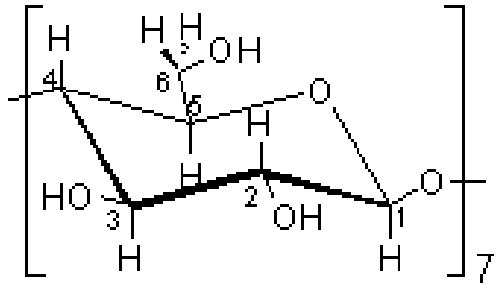
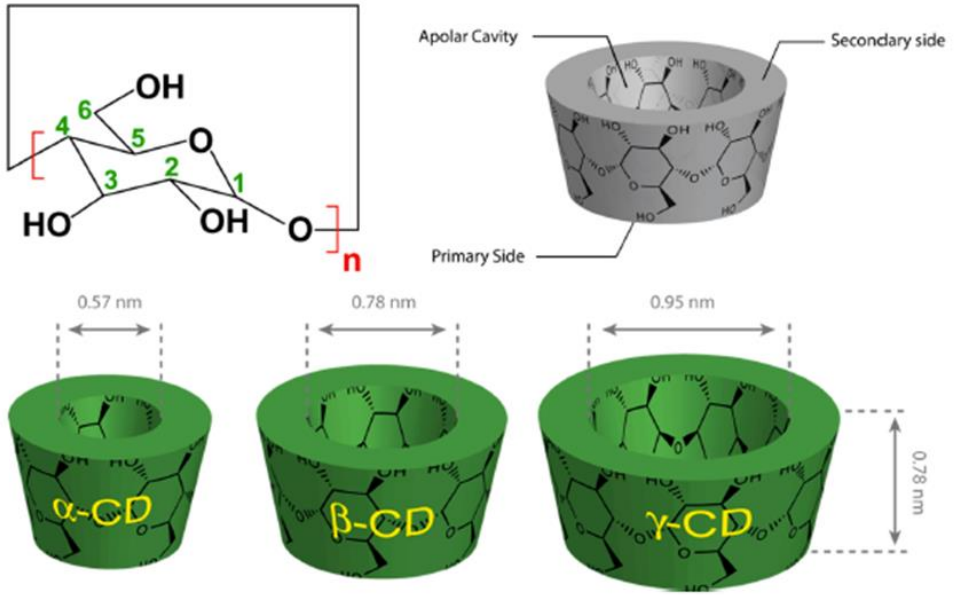


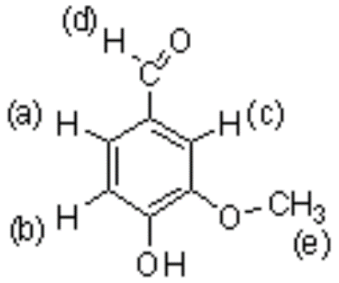
(a)



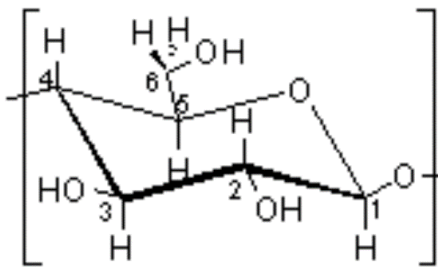
(b)



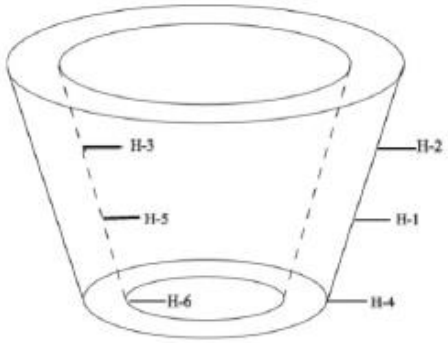
Top: Functional structural scheme of α-CD ($n = 6$), β-CD ($n = 7$), and γ-CD ($n = 8$). Bottom: Geometric dimensi



(a)



(b)



β-CD

Table 1. Chemical shifts of the proton of vanillin and β-CD in the free and complexed states.

Proton	δ_{free} (ppm)	$^{*}\delta_c$ (ppm)
H3	3.8907	3.7774
H5	3.7819	3.6125
Ha	7.4522	7.5179
Hb	6.9767	7.0817
He	3.8459	3.9215

$^{*}\delta_c = \delta_{free} - \Delta\delta_c$

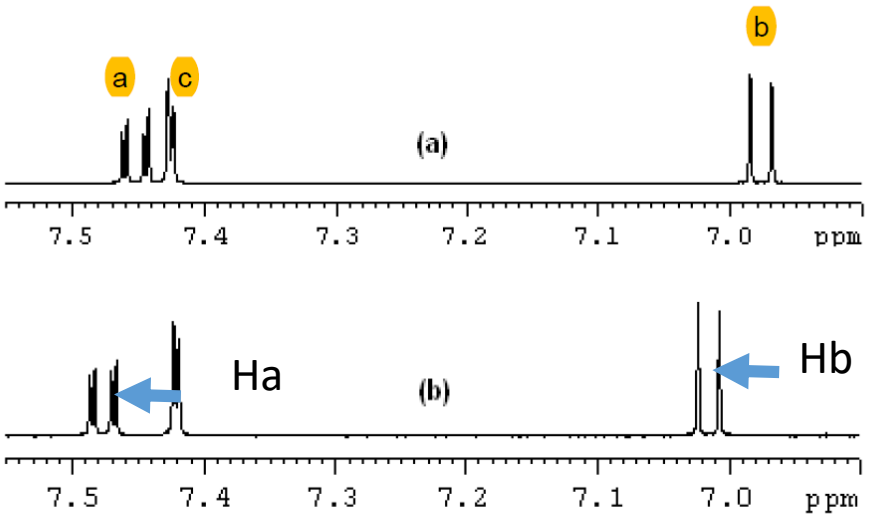


Figure 3. Partial ^{1}H NMR spectrum of (a) 10 mM vanillin and (b) 5mM vanillin and 5 mM β-CD.

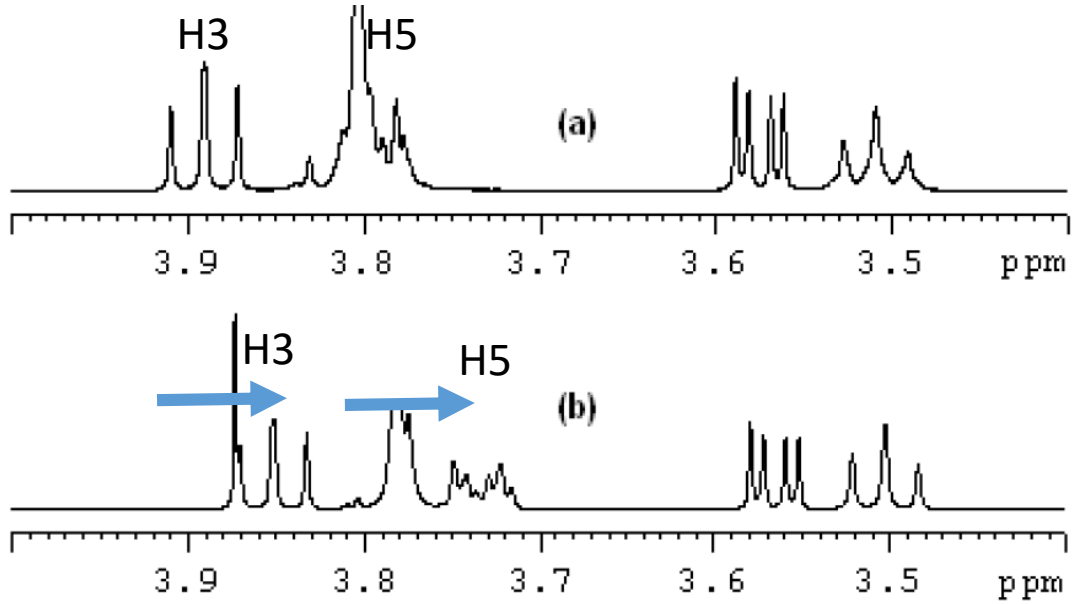


Figure 2. Partial ^{1}H NMR spectrum of (a) 10 mM β-CD and (b) 5 mM β-CD and 5 mM of vanillin.

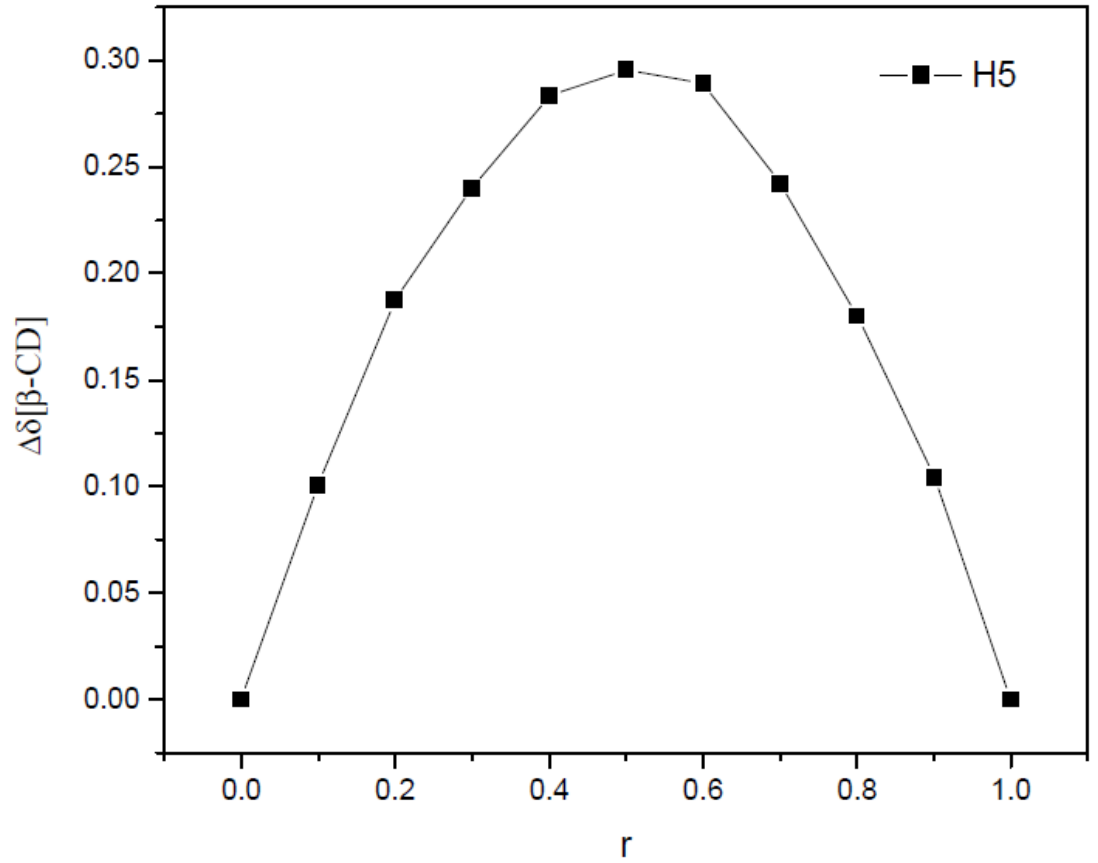


Figure 4. Concentrations variation plot for H-5 proton of β-CD.

The resulting continuous variation plots demonstrate that because r has a maximum value of 0.5 and highly symmetrical shapes (figure 4), the complex has 1:1 stoichiometry.

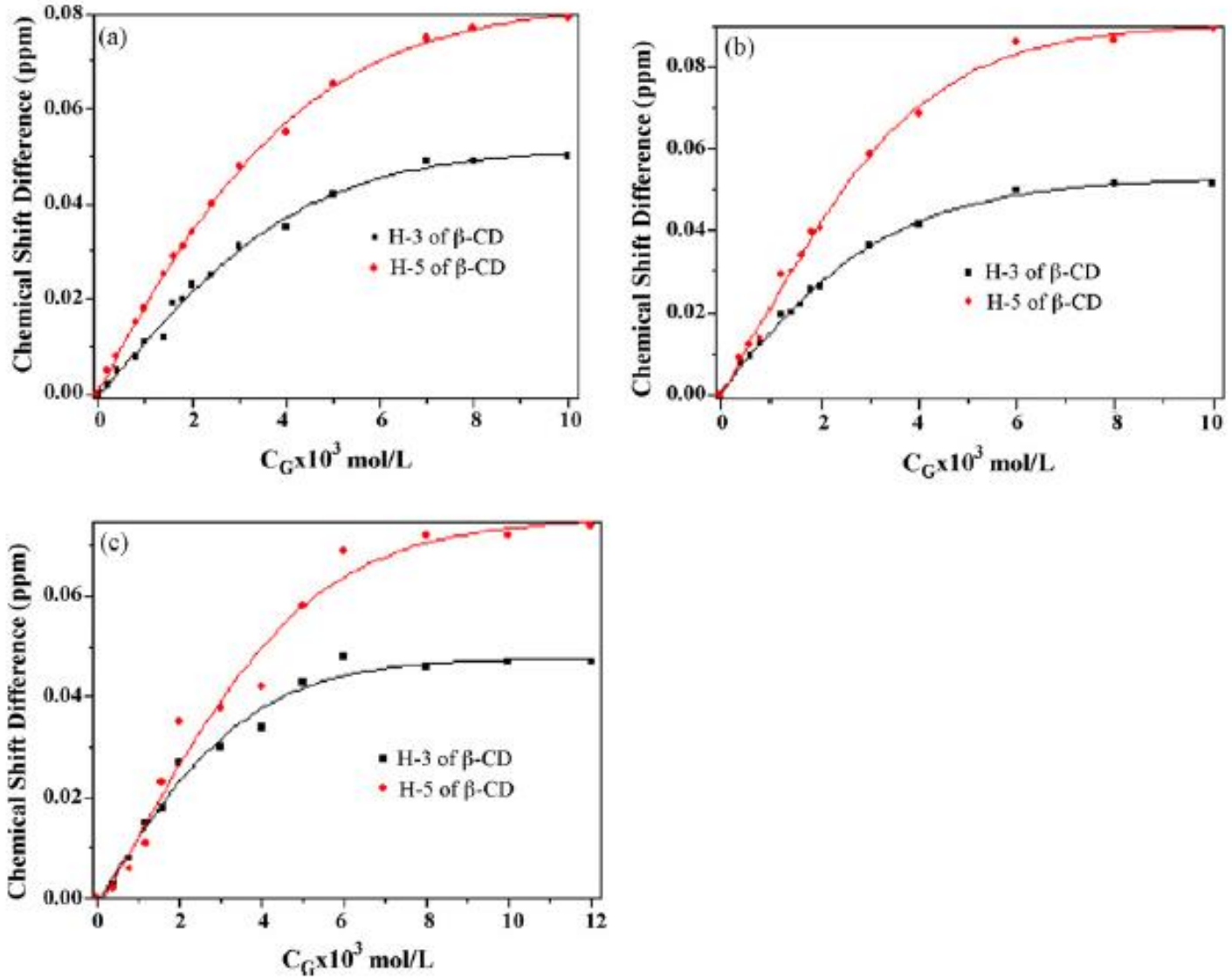
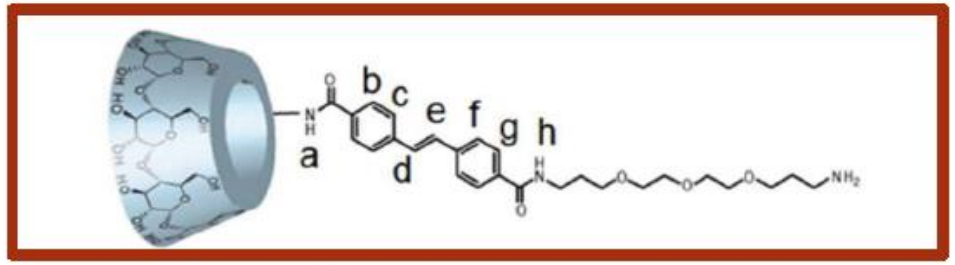
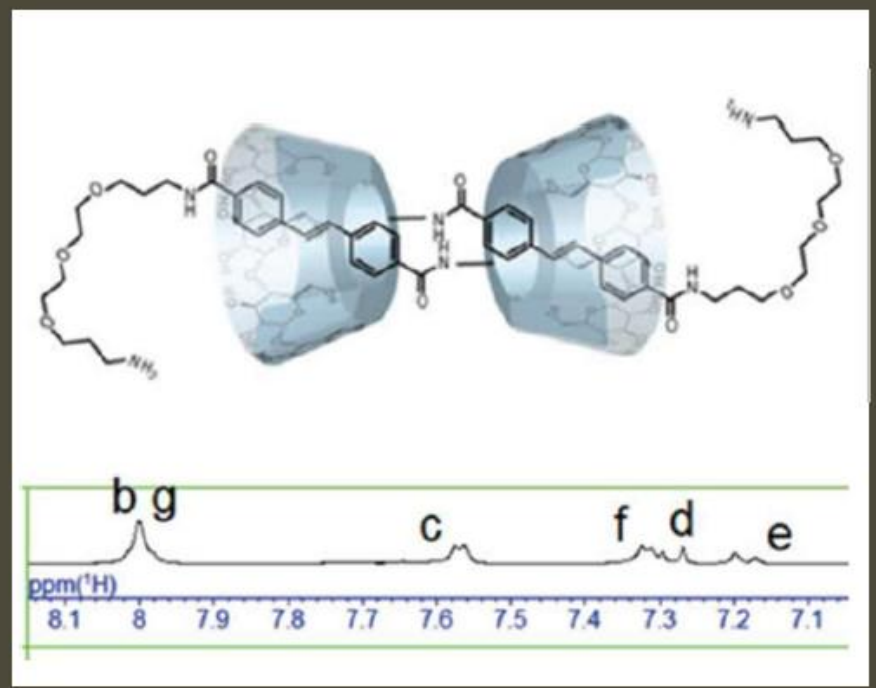


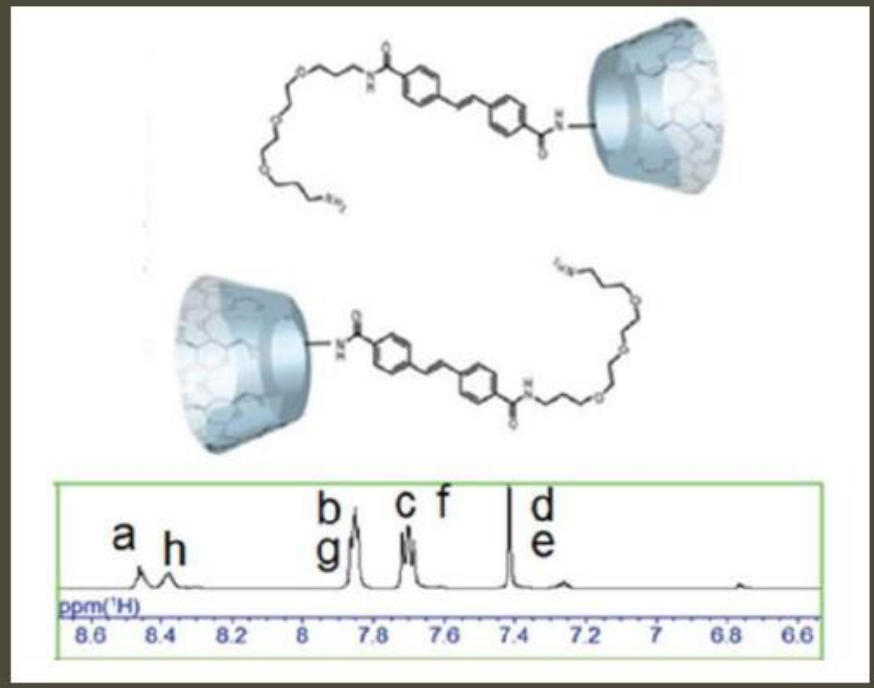
Fig. 4. The H5 and H3 chemical shift changes of β-CD plotted against the guest/host molar ratios with a fixed concentration of β-CD. (a) *cis*-cyclooctene +5.0 × 10^{-3} mol/L β-CD; (b) *cis*, *cis*-1, 3-cyclooctadiene + 5.0 × 10^{-3} mol/L β-CD; (c) *cis*, *cis*-1, 5-cyclooctadiene + 5.0 × 10^{-3} mol/L β-CD.

NMR analysis





aCD includes trans-Sti moieties to form $(aCD\text{-}Sti)_2$ complex in D_2O



$(aCD\text{-}Sti)_2$ complex is not formed in DMSO

Supramolecular Chemistry

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Directional Shuttling of a Stimuli-Responsive Cone-Like Macrocycle on a Single-State Symmetric Dumbbell Axle

*Jie-Shun Cui, Qian-Kai Ba, Hua Ke, Arto Valkonen, Kari Rissanen, and Wei Jiang**

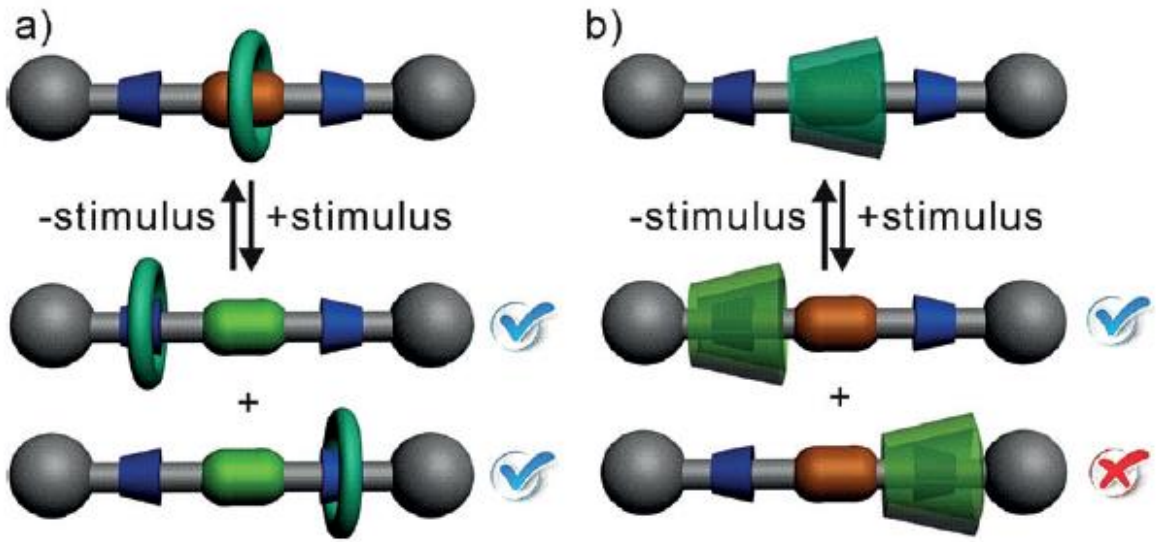


Figure 1. a) Molecular shuttle with a symmetric macrocycle and a multi-state symmetric axle; b) molecular shuttle with a stimuli-responsive cone-like macrocycle and a single-state symmetric axle.

Host: naphtotubes

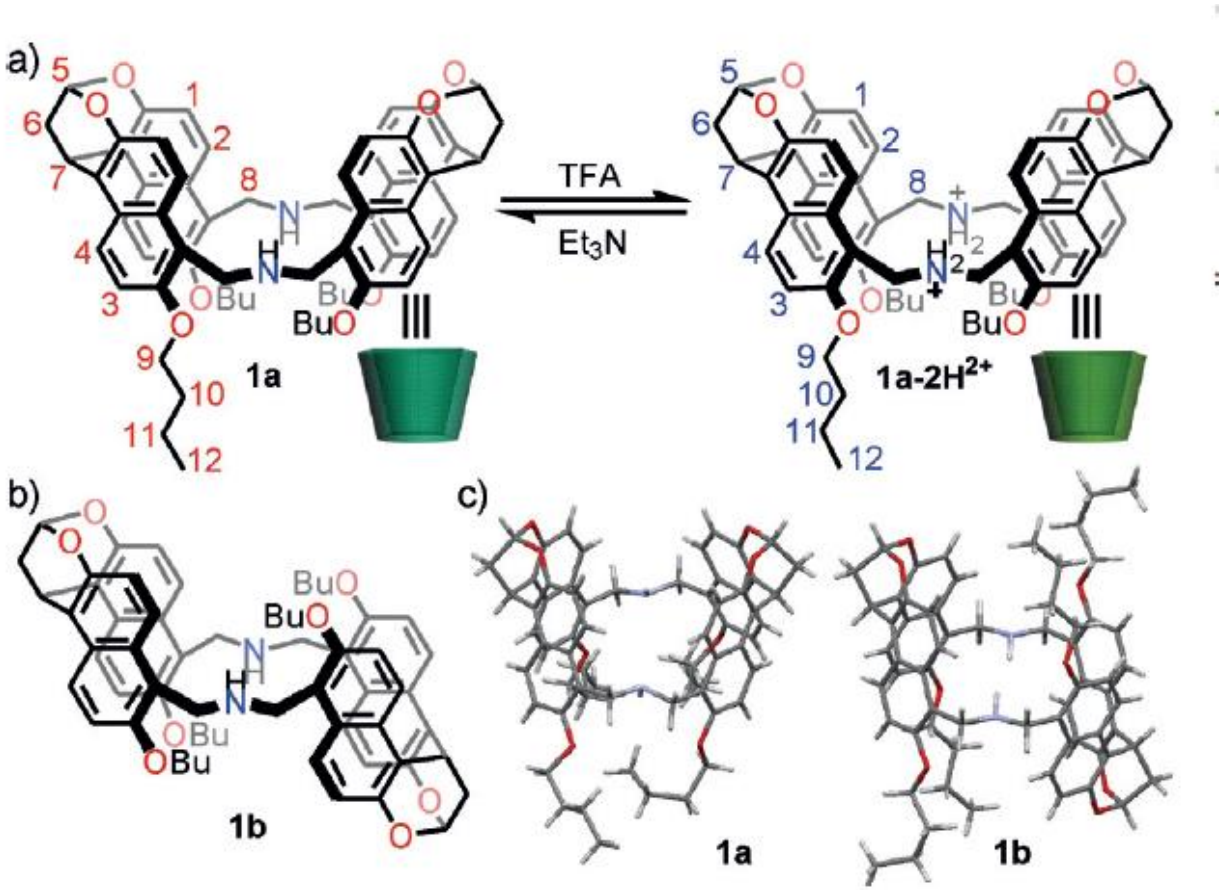
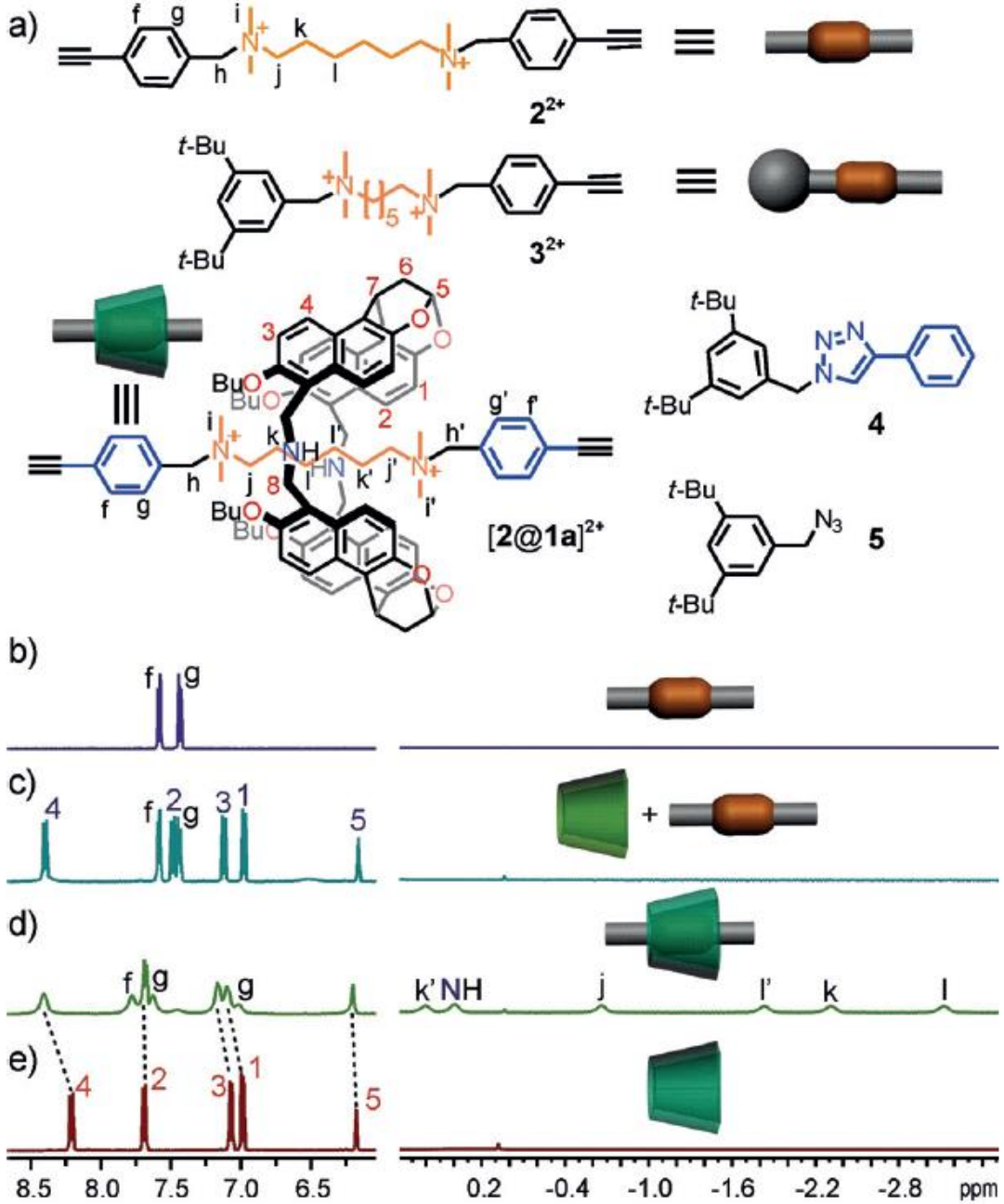


Figure 2. Chemical structures of a) **1a**, **1a**-$2H^{2+}$, and b) **1b**. The numberings on the structures were used to assign NMR peaks. c) Single-crystal structures of **1a** and **1b**. Solvent molecules were omitted for clarity.[18]


a)
2^{2+}
3^{2+}
$[2@1a]^{2+}$
4
5
b)
c)
d)
e)
8.5 8.0 7.5 7.0 6.5
0.2 -0.4 -1.0 -1.6 -2.2 -2.8 ppm

In laboratorio

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Laboratory Experiment

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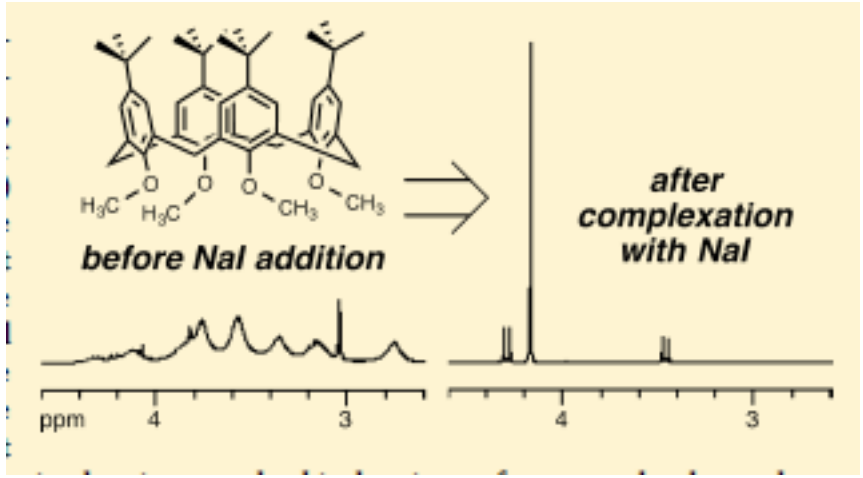
Synthesis and Characterization of Calixarene Tetraethers: An Exercise in Supramolecular Chemistry for the Undergraduate Organic Laboratory

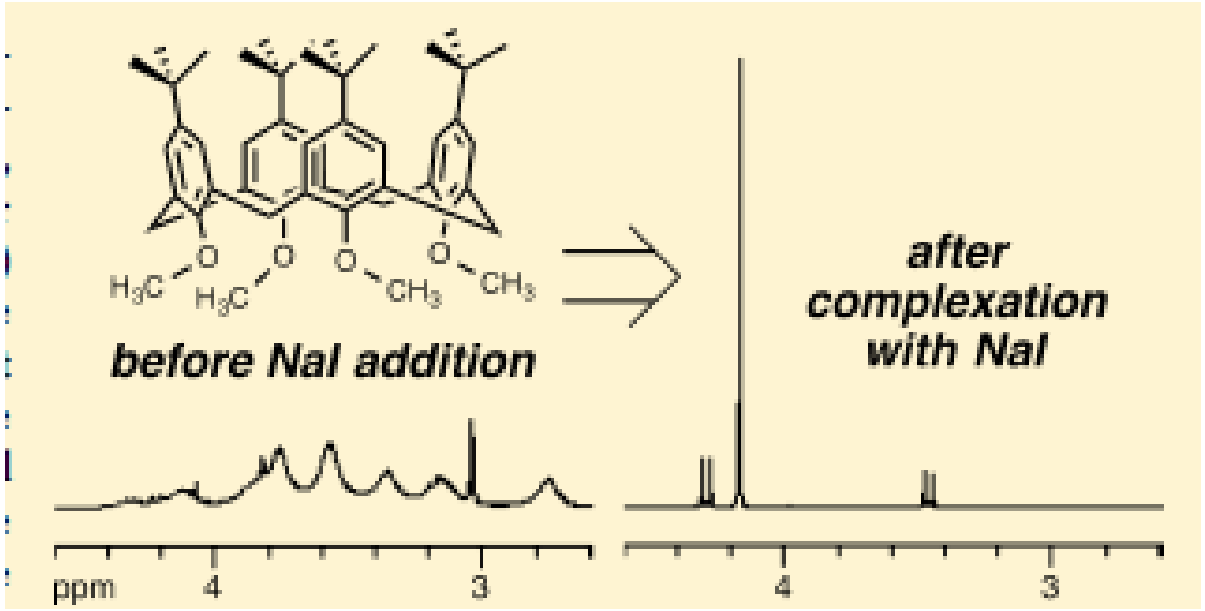
Stefan L. Debbert,* Bradley D. Hoh, and David J. Dulak

Department of Chemistry, Lawrence University, 711 East Boldt Way, Appleton, Wisconsin 54911, United States

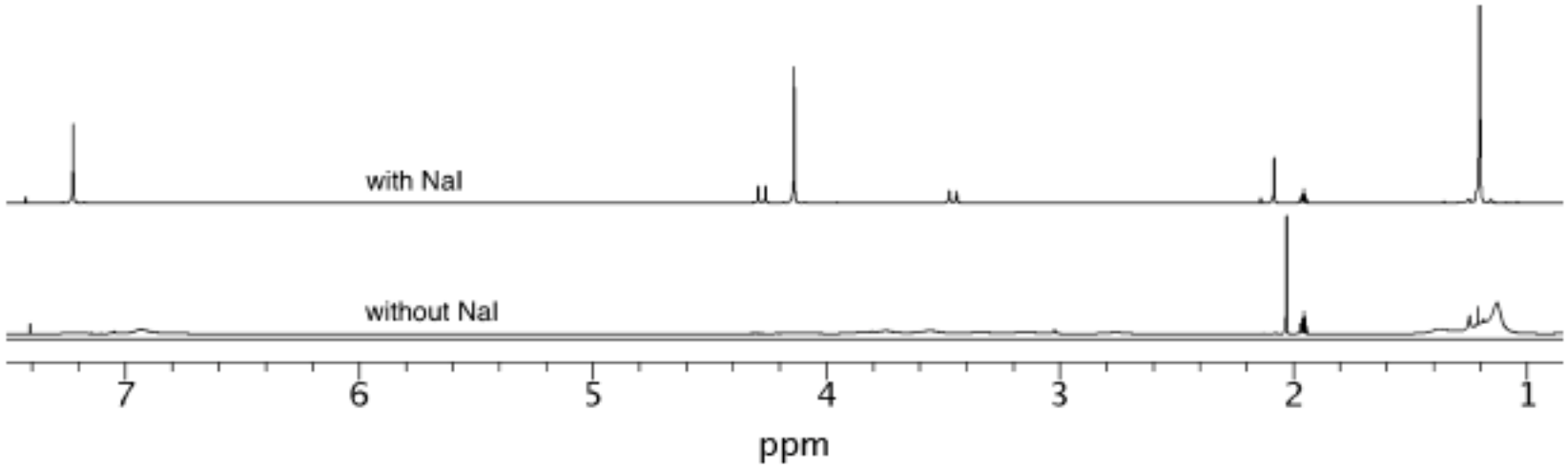
Supporting Information



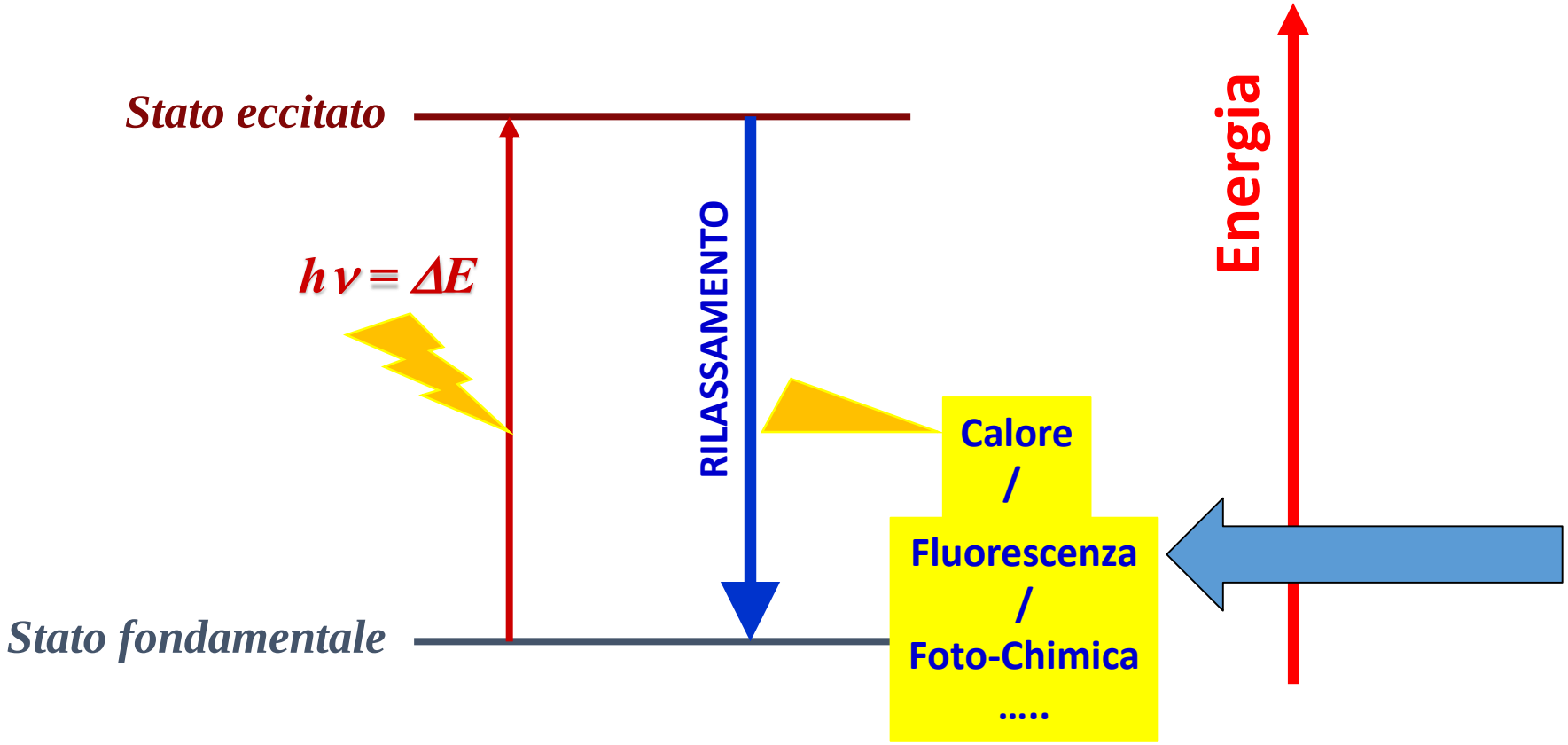
Synthesis (alkylation) and NMR spectra



In this case, upon addition of Na^+ cations and calix[4]arene complexation, the calix is conformationally blocked.



RILASSAMENTO

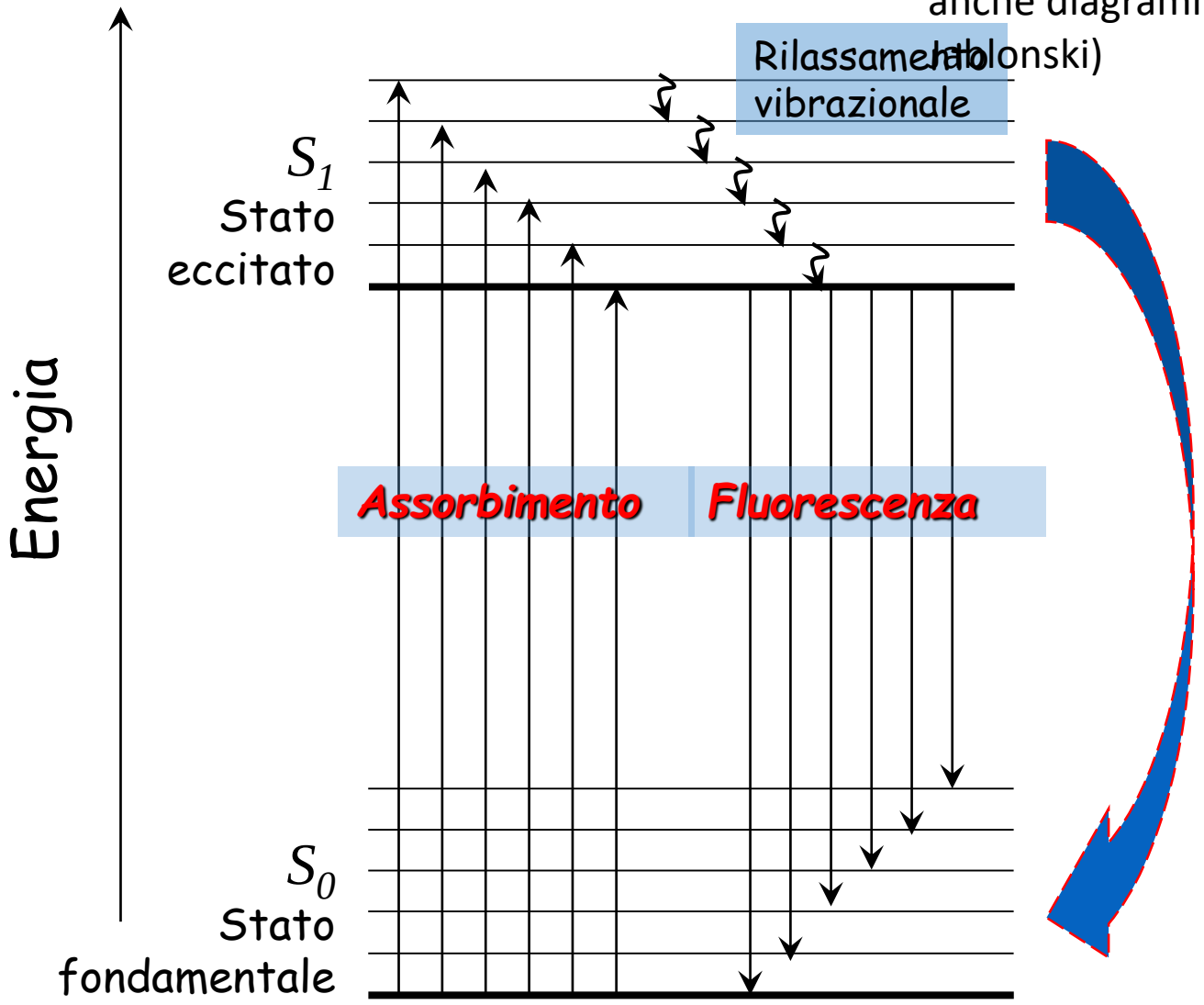


Molecola (M) + $h\nu$ (es. UV-Vis) → Molecola eccitata (M*)
M* ha una vita media da 10^{-8} a 10^{-9} s

M*→M + Energia (calore, radiazione …)

Fluorescenza

Diagramma dei livelli energetici (detto anche diagramma di Jablonski)

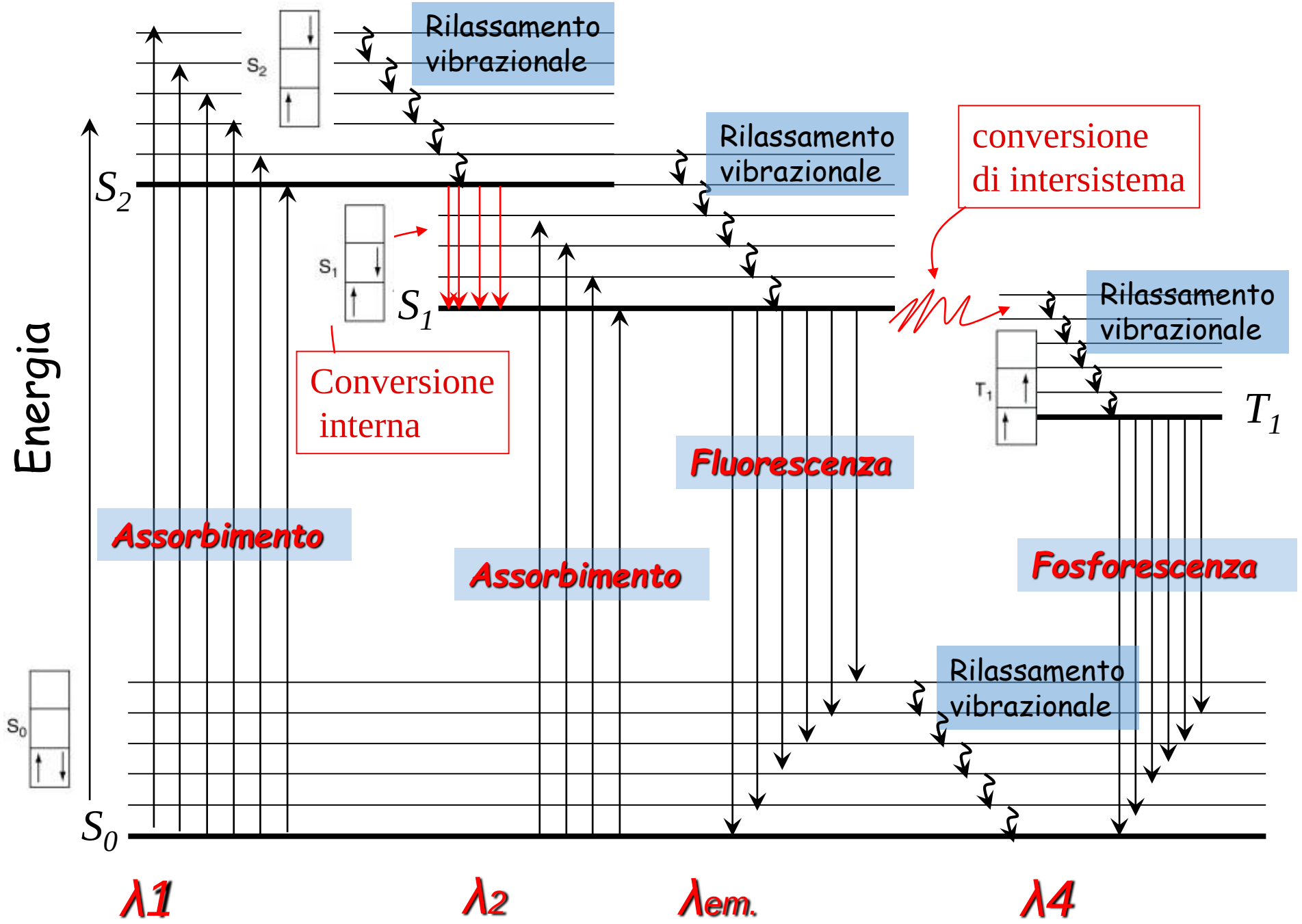


Rilassamento del fluoroforo dallo stato eccitato a quello fondamentale senza emissione di luce = Quenching*

*più avanti definiremo meglio il quenching

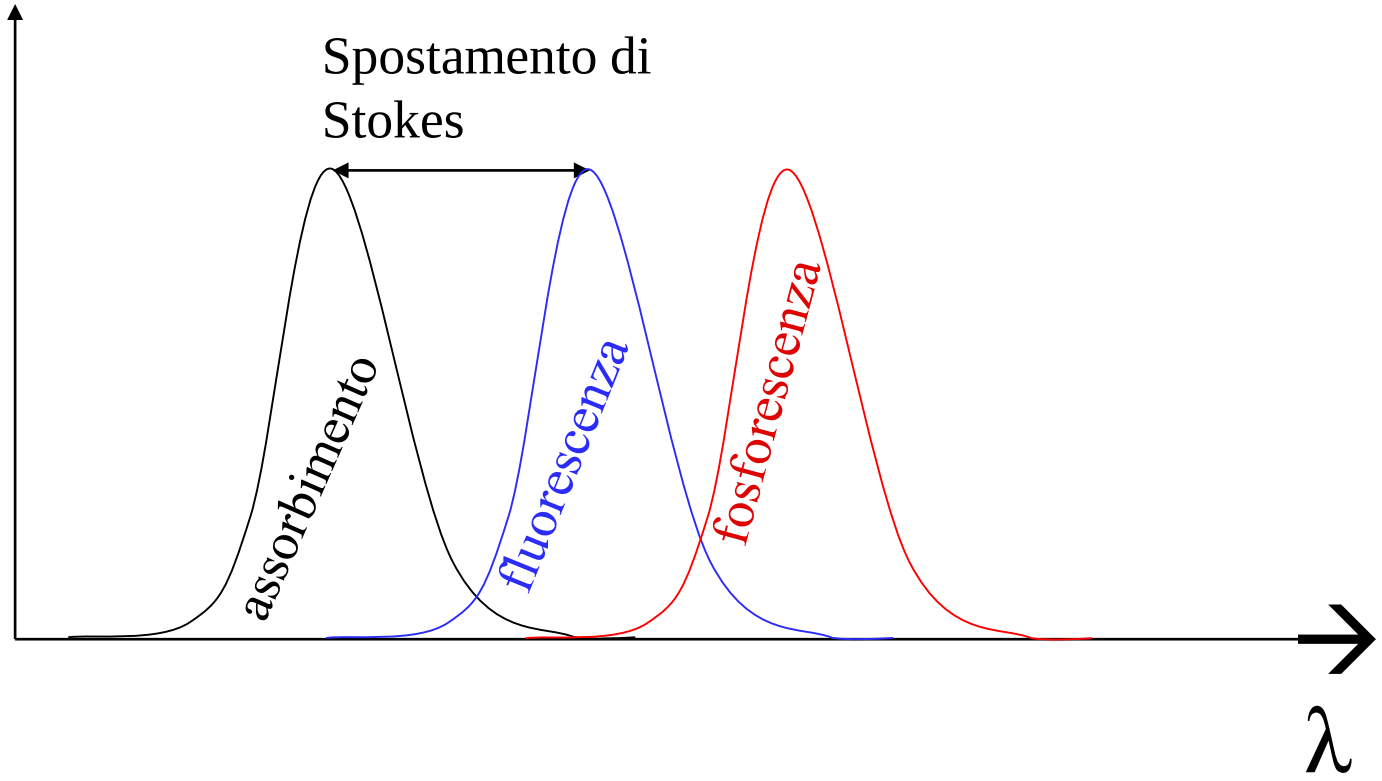
$\lambda_{ecc.}$ $\lambda_{em.}$

$$\text{Resa quantica} = \frac{\text{fotoni emessi}}{\text{fotoni assorbiti}}$$

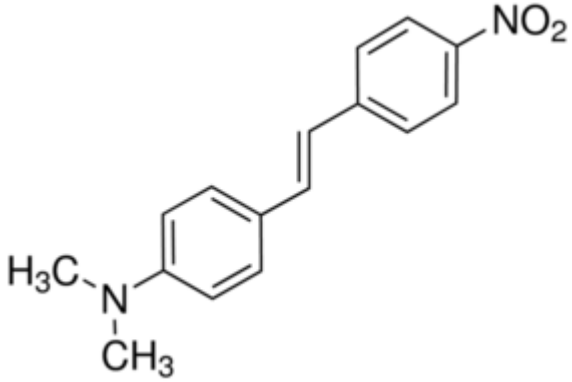

Rilassamento vibrazionale
Rilassamento vibrazionale
conversione di intersistema
Rilassamento vibrazionale
Conversione interna
Fluorescenza
Assorbimento
Assorbimento
Fosforescenza
Rilassamento vibrazionale
Energia
S_2
S_1
T_1
S_0
λ1
λ2
λem.
λ4

Conversione di intersistema→ Fosforescenza

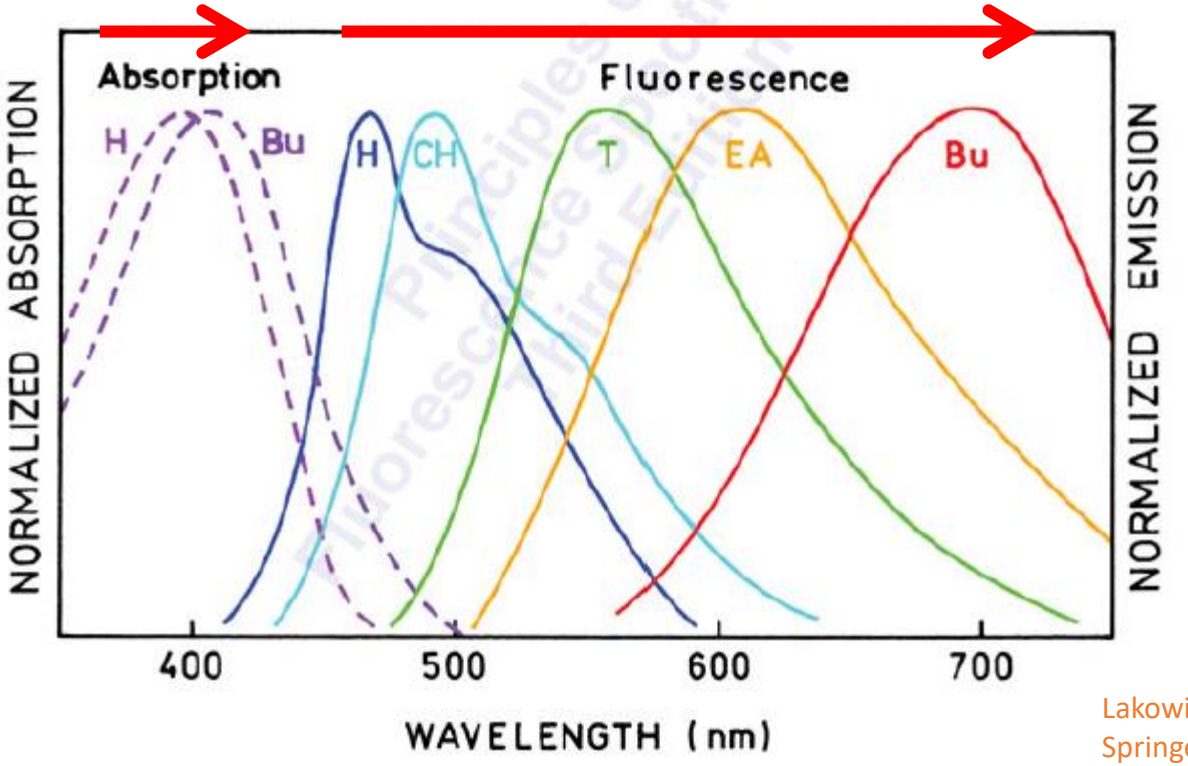
Lo stato di tripletto ha una energia piu‘ bassa dello stato eccitato di singoletto e la fosforescenza ha lunghezze d'onda maggiori della fluorescenza e puo' essere facilmente risolta.
La conversione di intersistema è favorita dalla presenza di atomi di iodio e bromo nella struttura chimica e dall’ossigeno molecolare



Effetto della polarità del solvente



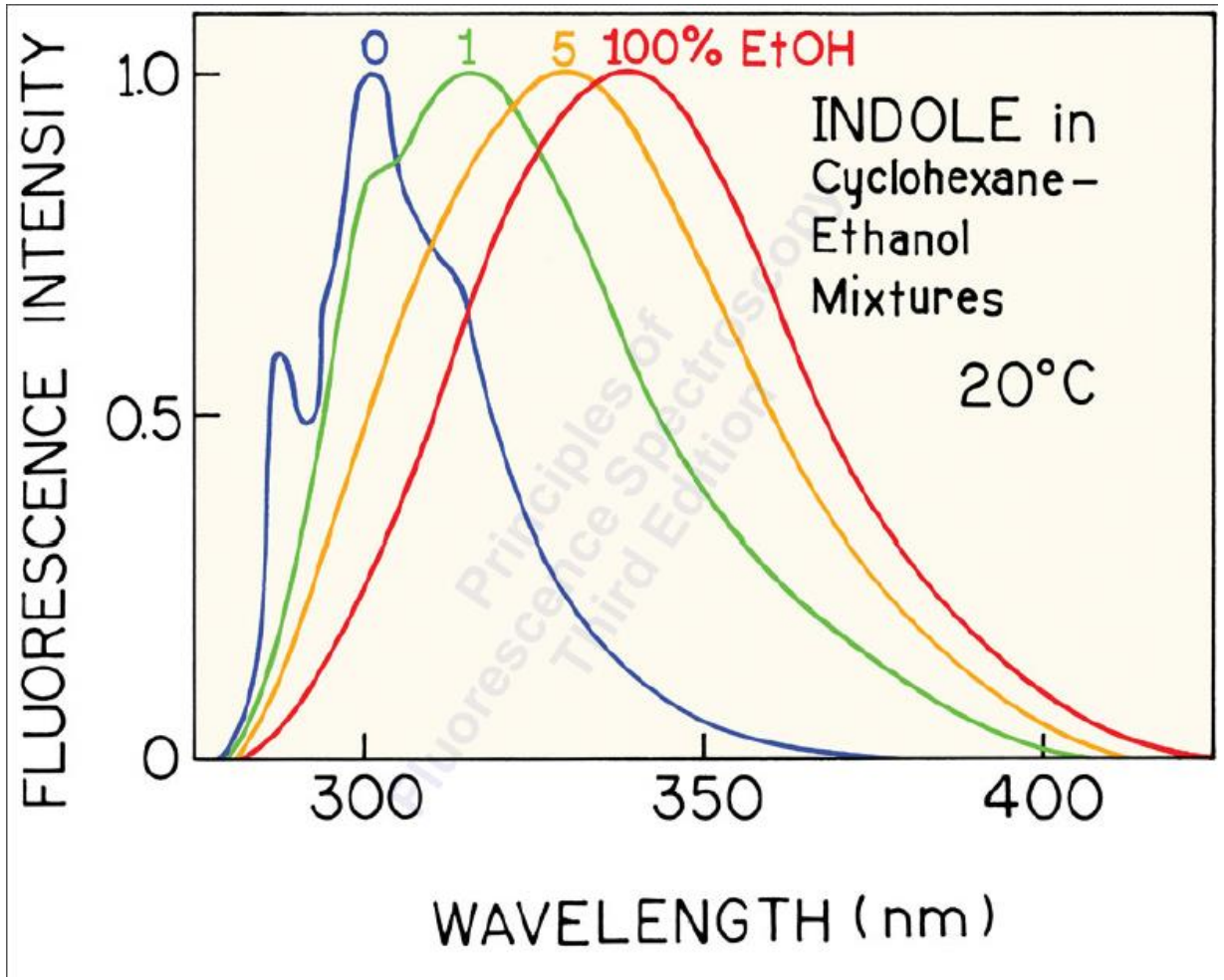
Assorbimento e emissione di **DNS (4-dimethylamino-4'-notrostilbenze**) in solventi a polarità crescente : H esano, CH cicloesano, T toluene, EA etil acetato, Bu n-butanolo.



→Effetto piccolo in Abs ma grande in Emissione

Lakowicz, Principles of Fluorescence Spectroscopy; Springer Science+Business Media, LLC

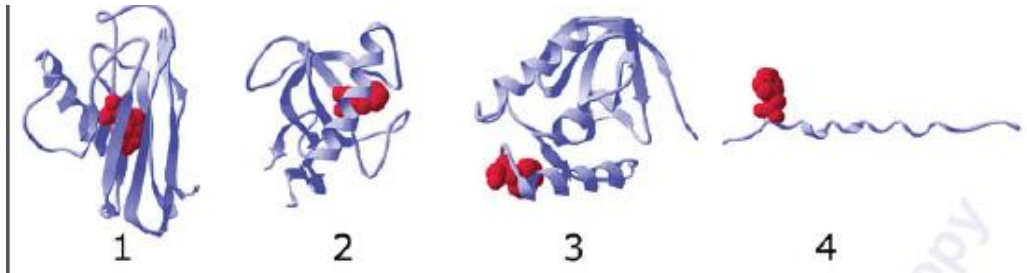
Non solo la polarità, ma anche altri fattori: ad esempio la capacità di fare ponti idrogeno



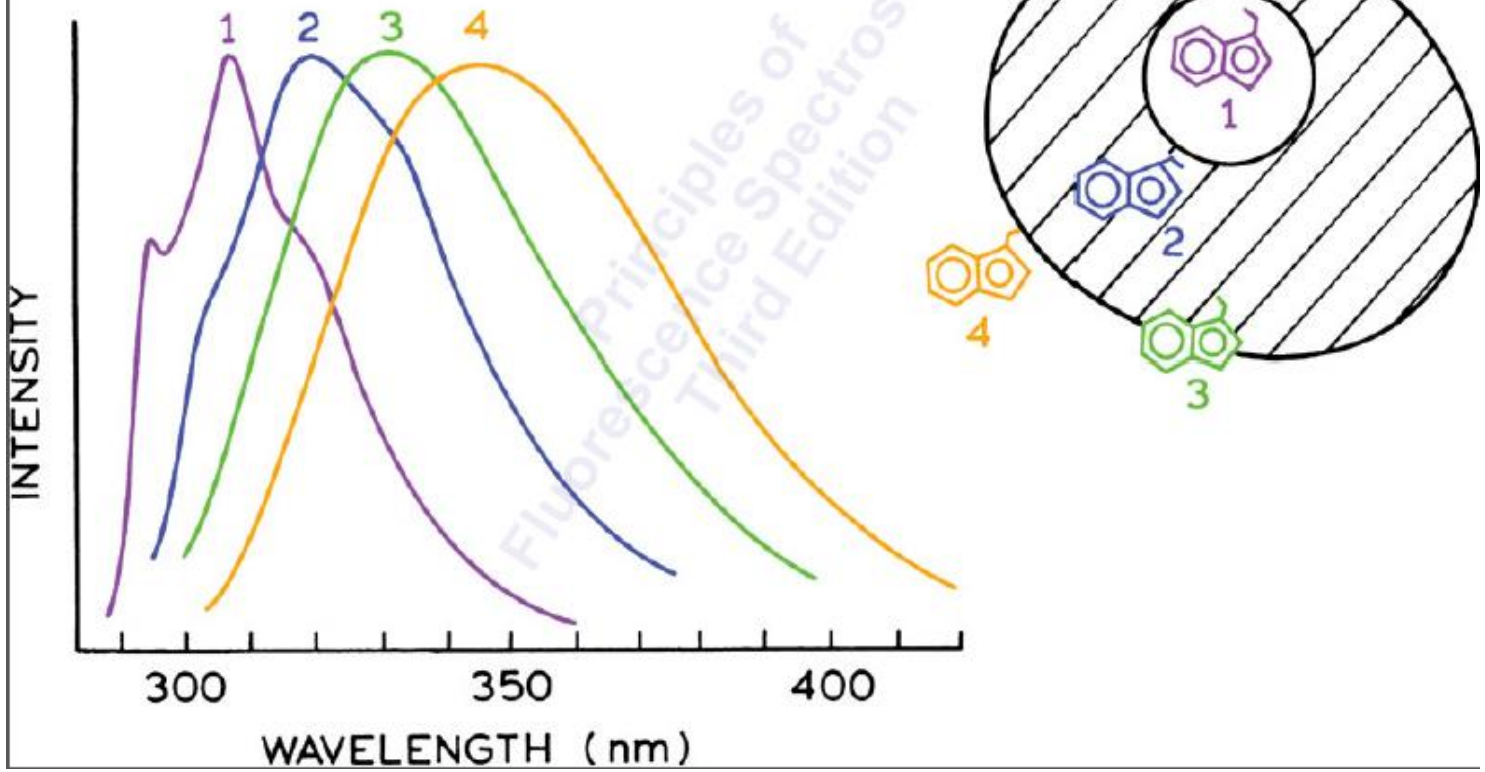
Spettro 0, indolo in cicloesano. Lo spettro di emissione è strutturato e sembra speculare a quello di assorbimento.
Nello spettro con 1% di EtOH lo spettro si sposta e modifica la sua forma a causa della capacità di fare ponti idrogeno con N imino dell'anello dell'indolo

Lakowicz, Principles of Fluorescence Spectroscopy; Springer Science+Business Media, LLC

Fluorescenza di proteine con un singolo Trp



1)Apoazurin Pfl;
2) ribonuclease T1;
3) staphylococcal nuclease;
4) glucagon



Lakowicz, Principles of Fluorescence Spectroscopy; Springer Science+Business Media, LLC

Effetto sulla fluorescenza di

→ Temperatura:

L'intensità di fluorescenza tipicamente diminuisce all'aumentare della temperatura poiché aumenta l'agitazione termica e le collisioni tra i fluorofori e con il solvente. Il risultato è una maggiore conversione interna ed esterna.

→ Viscosità della soluzione:

In genere se la viscosità aumenta, aumenta anche l'intensità fluorescenza (per le stesse ragioni viste per la temperatura; inoltre per alcuni fluorofori aumenta la rigidità strutturale).

→ pH:

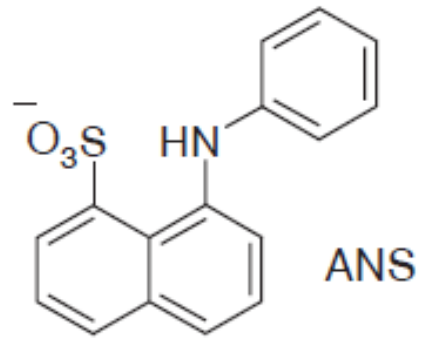
Può cambiare la struttura delle diverse forme di risonanza in gruppi fluorofori con sostituenti acidi o basici. Può modificare sia l'intensità di fluorescenza che la lunghezza d'onda di eccitazione ed emissione.

→ L'intorno chimico/fisico del fluoroforo (vedi Trp e ANS per le proteine)

→ La presenza di molecole quenchers (vedi dopo):

- Lo stato eccitato non si forma (q. Statico) o viene depopolato (q. Dinamico) per vie alternative alla fluorescenza

A simple plot of F_o/F against [H] from titration of the quenching host into a guest solution should yield a straight line of slope K_{11}. Common fluorescent guests such as 8-anilino-1-naphthalenesulfonate (ANS, **1.7**) may be used to probe complexation ability of various hosts in this way.

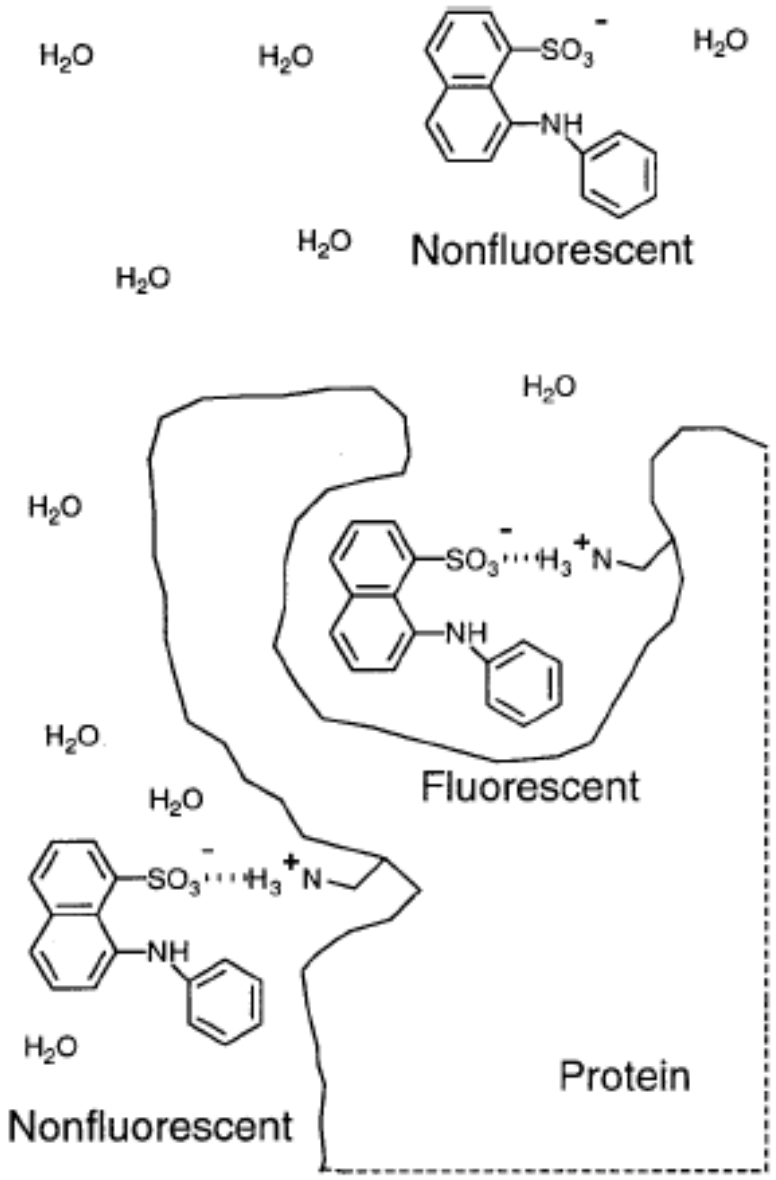


ANS

1.7

A Visual Demonstration of Supramolecular Chemistry: Observable Fluorescence Enhancement upon Host–Guest Inclusion

ANS (1 Anilino-8 Naphthalene Sulfonate)



Assorbimento λ max = 374 nm
Emissione λ max = 454 nm

→ In presenza di regioni idrofobiche accessibili (ad es. in intermedi del folding) la resa quantica di fluorescenza aumenta

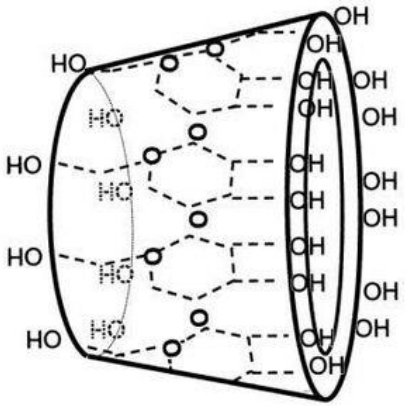
$$\text{Resa quantica} = \frac{\text{fotoni emessi}}{\text{fotoni assorbiti}}$$

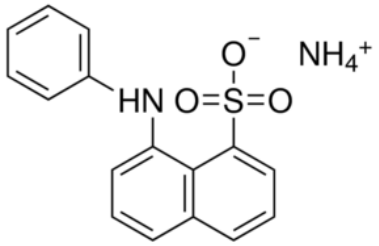
Fluorescence enhancement

In free aqueous solution, ANS does not fluoresce significantly, but when included inside the cyclodextrin cavity, the probe becomes highly fluorescent. ANS

fluorescence was found to be enhanced by factors of 8.4, 100, and 180 upon addition of 10 mM β-cyclodextrin, methyl-β- cyclodextrin, and hydroxypropyl-β-cyclodextrin (HP-β-CD),

respectively. In the latter two cases, the enhancements are so large that they are clearly visible to the naked eye, using a simple hand-held UV lamp as the excitation source.





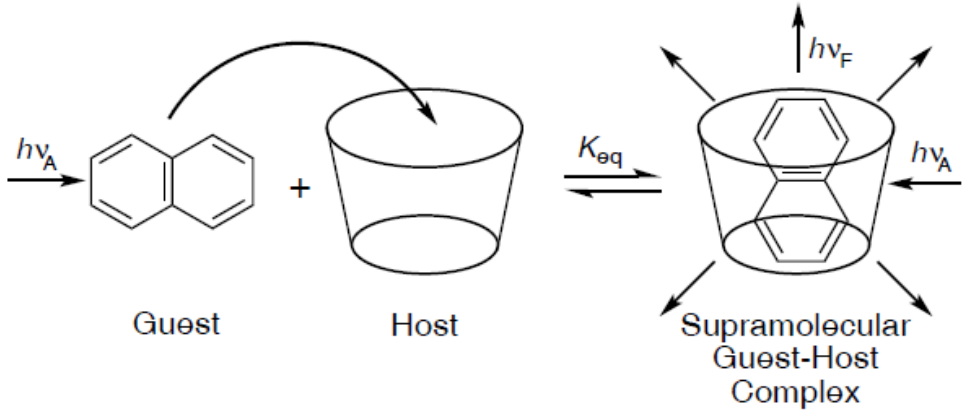
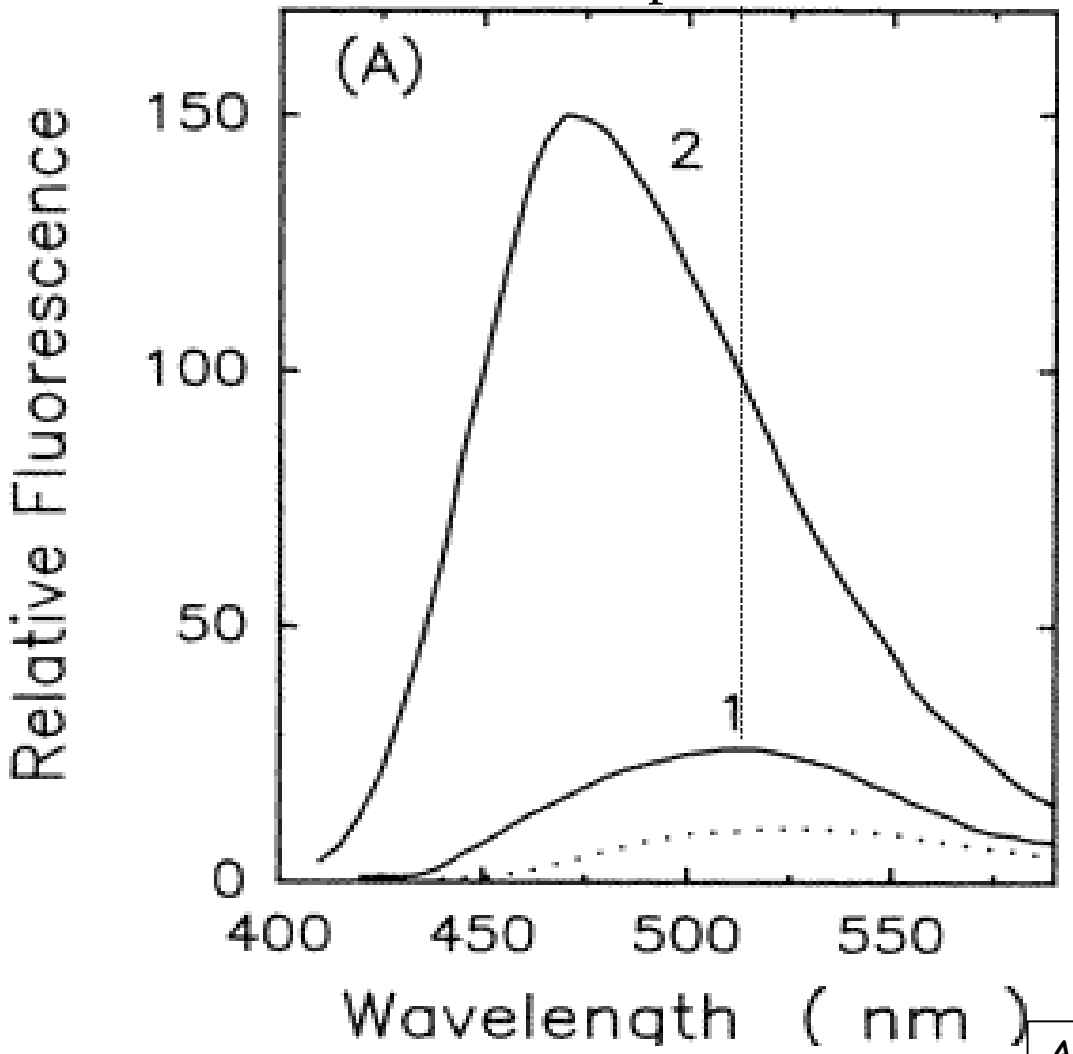


Figure 2. A pictorial representation of the formation of the host–guest complex of a naphthalene-based fluorescent probe, such as ANS, and a cyclodextrin.

Spectrum of ANS in the presence of β-lactamase under native (1) and partially denaturing (2) conditions; dotted lines correspond to the spectrum of ANS in the absence of protein.



When ANS binds to the protein's exposed hydrophobic patches, its fluorescence quantum yield
is strongly enhanced and its emission maximum is
shifted to lower wavelengths.

Assorbimento λ max = 374 nm
Emissione λ max = 454 nm

Fluorescence titrations

- Fluorescence titration experiments are based on the proportion of fluorescence intensity to fluorophore concentration (concentration of fluorescent species in solution, this is often a fluorescent guest, G)
- 1:1 stoichiometry of the complex
- Either the guest or the host-guest complex are non-fluorescent, in this case the host acts as a quencher of fluorecence.
- A simple plot of F_0/F against [H] from titration of the quenching host into a guest solution should yield a straight line of slope K_{11}

$$F_0/F = 1 + K_{11}[H]$$

Fluorescence titration measurements are based on the proportion of fluorescence intensity to fluorophore concentration (concentration of fluorescent species in solution; this is often a fluorescent guest, G). For a 1:1 complex with host, H, with stability constant K11 = [HG]/[H][G] the fluorescence intensity F is given by: F = kG [G] + k11 [HG]

$$\frac{F}{F_o} = \frac{k_G/k_G^o + (k_{11}/k_G^o)K_{11}[H]}{1 + K_{11}[H]} \qquad (1.15)$$

This equation is greatly simplified for cases where either the guest or host–guest complex are non-fluorescent (*i.e.* the fluorescence is 'turned on' by complexation, or in the case of quenching by the host), in which case either k_G or k_{11} become zero. For example, for $k_G = k_G^0$ and $k_{11} = 0$, we obtain:

$$\frac{F_o}{F} = 1 + K_{11}[H] \qquad (1.16)$$

Static quenching

The quencher molecule (HOST) interacts with the fluorophore (GUEST) in its ground state (not excited) and forms a non-fluorescent complex

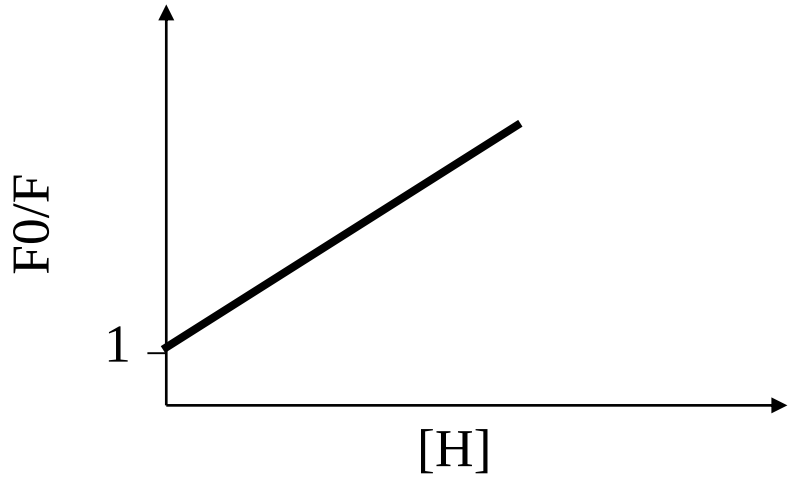
Fluorescence titration (static quenching)

→ Is described by the equation:

$$F_0/F = 1 + K_{11}\,[Q] \text{ (or } [H])$$

where K11 is a constant referred to quencher/fluorophore interaction

The F_0/F Vs [H] is a linear plot with slope K_{11}



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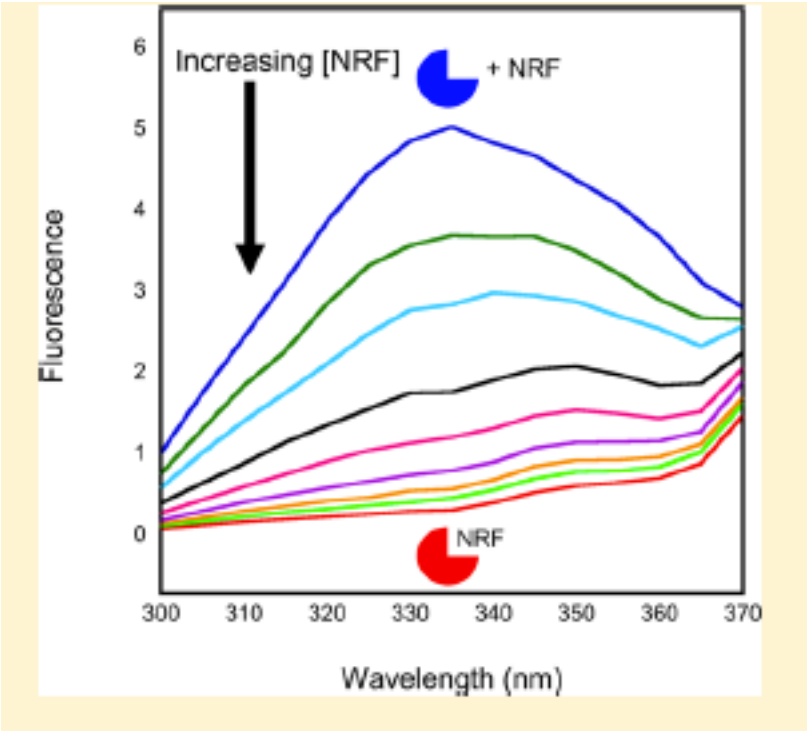
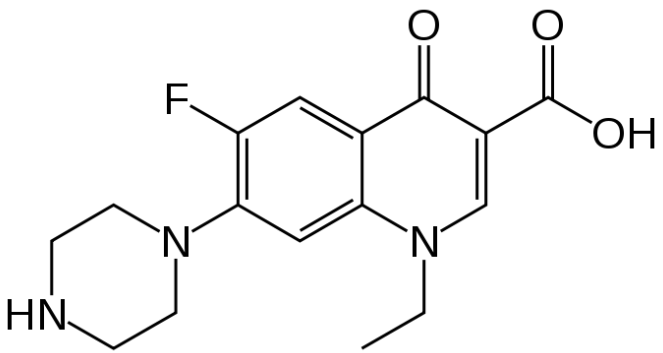
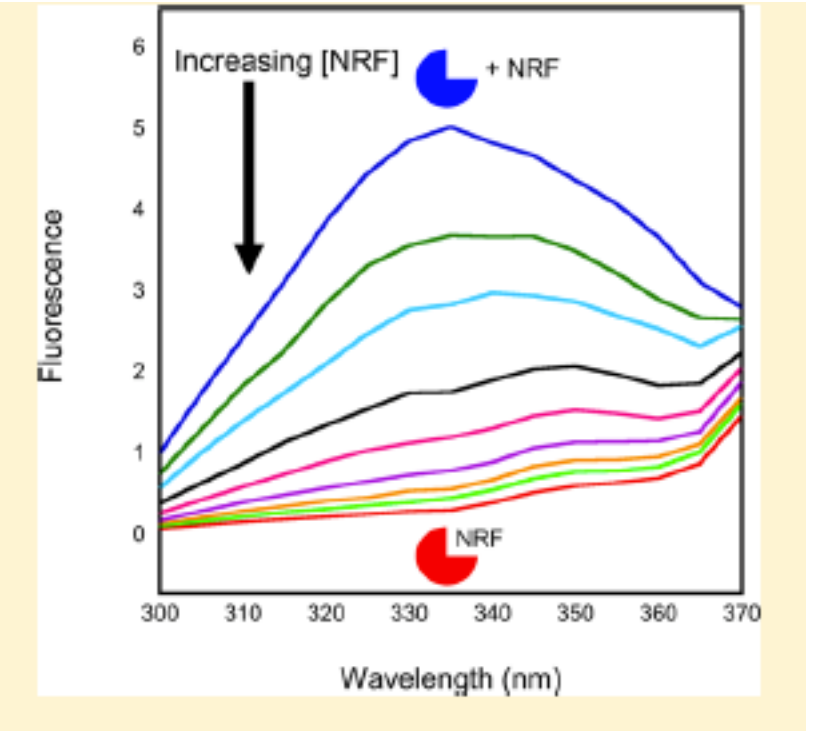
Measuring Norfloxacin Binding to Trypsin Using a Fluorescence Quenching Assay in an Upper-Division, Integrated Laboratory Course

Katherine A. Hicks*



ABSTRACT: Fluorescence quenching assays are often used to measure dissociation constants that quantify the binding affinity between small molecules and proteins. In an upper-division undergraduate laboratory course, where students work on projects using a guided inquiry-based approach, a binding titration experiment at physiological pH is performed to analyze the interaction between the drug norfloxacin and the protein trypsin. The resulting nonlinear binding data are fitted using a scientific data analysis program to determine the dissociation constant (K_d value) describing this interaction. The experiment is especially of interest to students who plan to pursue careers in health-related aspects of biochemistry.





Determination of Binding Constants

The adjusted fluorescence emission at 335 nm is plotted as a function of NRF concentration. In the lab handout, students are provided with a modified direct binding eq (eq 1) that is used to calculate the K_d value.[16]

$$\Delta Fl = \frac{IF}{1 + [NRF]/K_d} + EP \quad (1)$$

where ΔFl is the change in the adjusted fluorescence, IF is the initial fluorescence, EP is the endpoint fluorescence, [NRF] is the concentration of NRF, and K_d is the dissociation constant describing the binding between NRF and trypsin. The derivation to this equation is provided in Supporting Information Section 7. We used the program KaleidaGraph (Synergy Software) for these analyses, which provides students with exposure to nonlinear fitting programs that they will encounter if they continue in the field. Other curve fitting programs that can fit arbitrary functions include SigmaPlot (Systat Software) and Prism (GraphPad Software), which could also be used for these analyses.

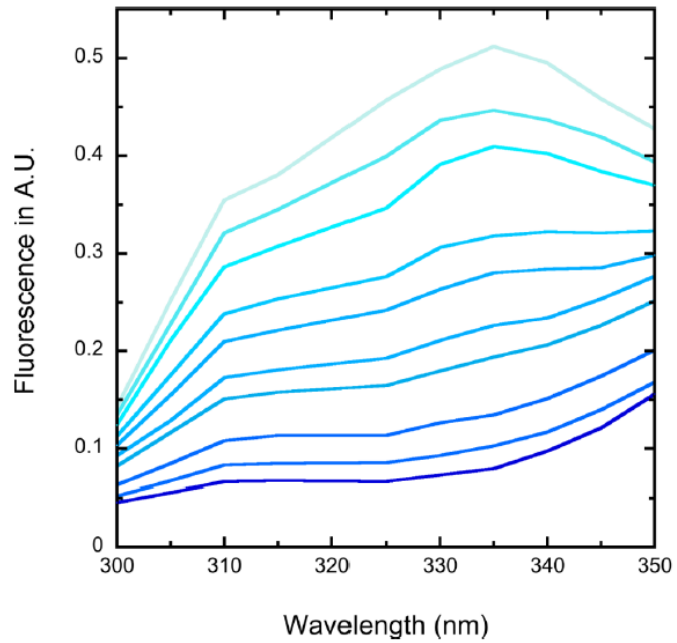


Figure 1. Emission spectra of the NRF binding experiment. NRF was titrated into a cuvette containing 250 nM trypsin and 50 mM Tris (pH 7.0). The intensity of the color corresponds to increasing NRF concentrations from 0 to 30 μM.

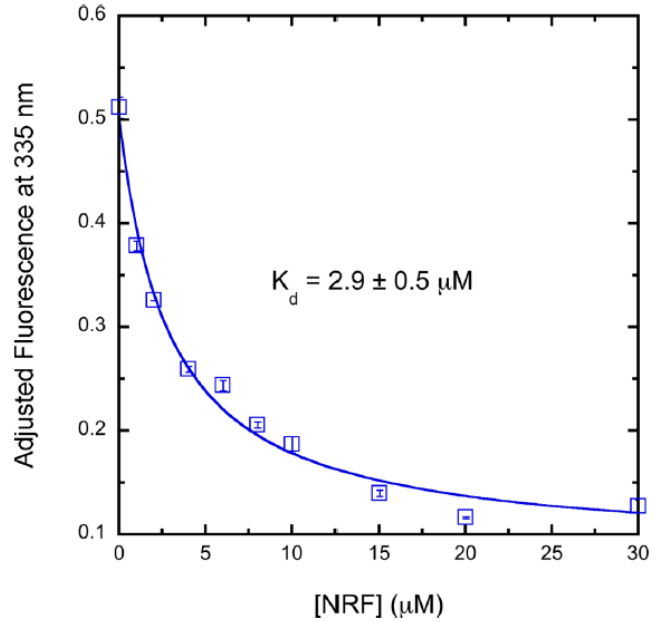


Figure 2. Measurement of K_d for the trypsin·NRF complex using fluorescence quenching. The assay contained 50 mM Tris (pH 7.0) and 250 nM trypsin. The sample was titrated with NRF (0–30 μM). The K_d value quantifying the interaction was determined by a fit of eq 1 to these data.

Quenching Dinamico-Collisionale

→La molecola quencher interagisce con il fluoroforo durante il tempo di vita dello stato eccitato (10^{-5}-10^{-10} s), che si rilassa per via non radiativa.

→Quindi lo stato eccitato viene depopolato con riduzione dell'intensità di florescenza.

→Anche il lifetime di fluorescenza diminuisce poiché aumenta la velocità di depopolamento dello stato eccitato attraverso processi non radiativi.

$$F_0/F = \tau_0/\tau$$

con F_0; F e τ_o; τ intensità di fluor. e lifetime in assenza (F0; τo) e in presenza del quencher.

In molti libri e articoli i due termini, dinamico e collisionale, vengono usati come sinonimi. Il qu. Collisionale è un esempio di qu. Dinamico. Anche altri processi (quali la FRET) possono esser considerati esempi di qu. Dinamico. Generalmente la FRET non viene trattata come esempio di qu. Dinamico.

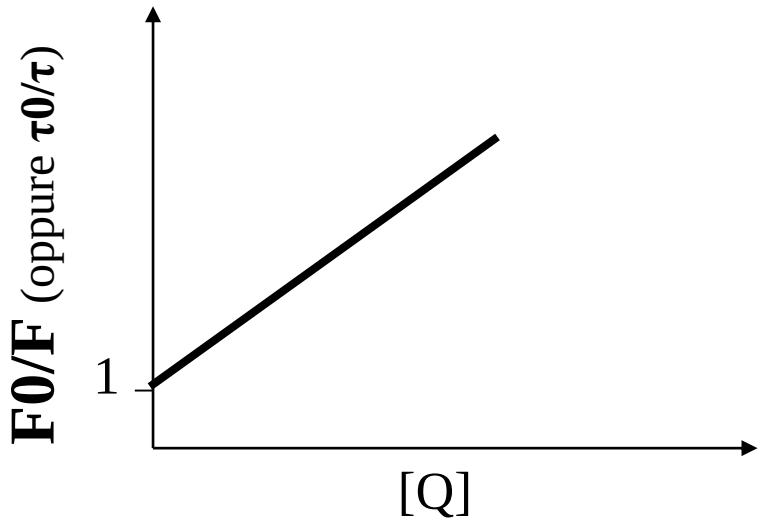
Quenching Dinamico-Collisionale

- è descritto dall'equazione di STERN-VOLMER:

$$F_0/F = 1 + K_D [Q]$$

K_D costante di quenching SV (spesso indicata come K_{SV})

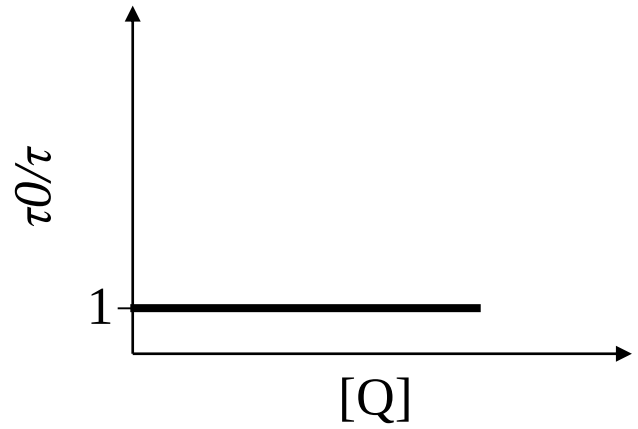
Il grafico F0/F Vs [Q] è lineare (se c'è una sola classe di fluorofori) con intercetta 1 e pendenza K_D



Quenching Statico

→ La molecola quencher interagisce con il fluoroforo nel suo stato fondamentale (non eccitato) formando un complesso NON FLUORESCENTE.

→Poichè non interagisce con lo stato eccitato non influenza τ ma solo riduce l'intensità di fluorescenza:



Quenching Statico e Dinamico-Collisionale si possono distinguere sulla base di:

→ τ_0/τ : è uguale ad 1 nel Q.Statico ; è funzione di [Q] nel Q.Dinamico-Collisionale.

→Dal confronto degli spettri di assorbimento del fluoroforo in presenza e assenza di Q. Infatti nel Q. collisionale non c'è interazione con lo stato fondamentale del fluoroforo che, quindi, non cambia il suo assorbimento. Nel Q.Statico, invece, ci può essere una variazione dello spettro di assorbimento.

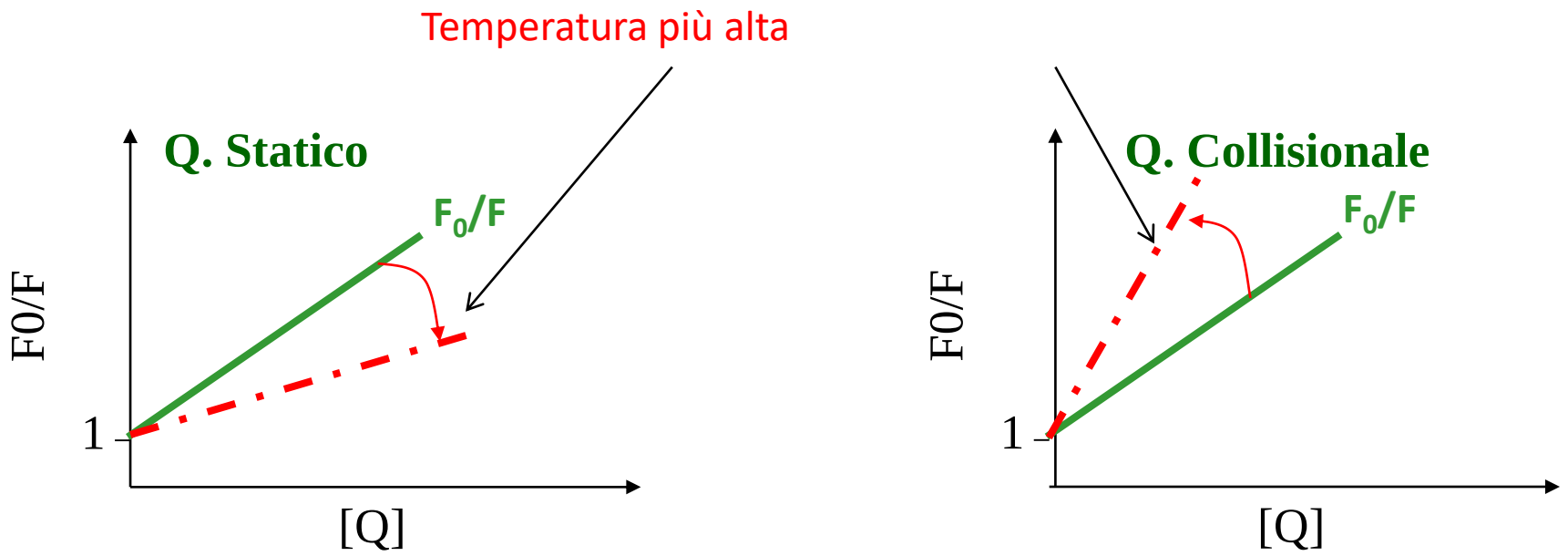
Quenching Statico e Dinamico-Collisionale si possono distinguere sulla base di:

→ Dalla dipendenza con la temperatura e la viscosità della soluzione.

- Il Q. Dinamico-Collisionale è strettamente legato alla diffusione del quencher, quindi aumenta con la temperatura e si riduce con la viscosità del mezzo.

- Il Q. Statico è legato alla costante di associazione del quencher con il fluoroforo non eccitato. All'aumentare della temperatura rompo il complesso è riduco il quenching.

F0/F *vs* [Q] a temperatura più alta



Quenching Statico e Dinamico-Collisionale si possono distinguere sulla base di:

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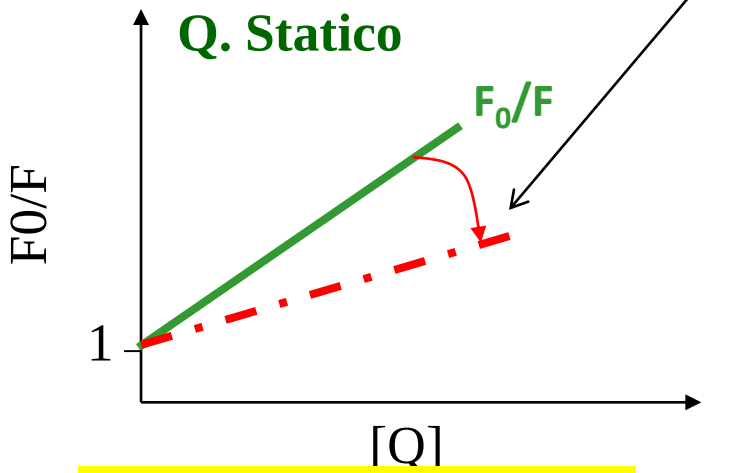
- Il Q. Statico è legato alla costante di associazione del quencher con il fluoroforo non eccitato. All'aumentare della temperatura rompo il complesso è riduco il quenching.

F0/F *vs* [Q] a temperatura più alta

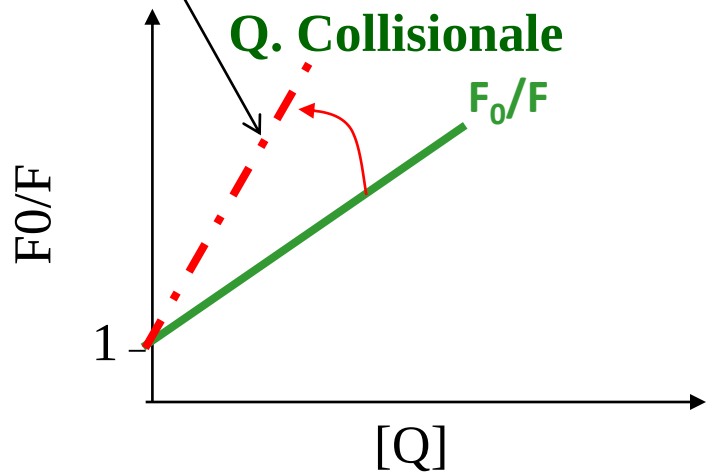
Temperatura più alta

$F_0/F = 1+K_? [Q]$



Pendenza= Ks*=$^{[FQ]}/_{[F][Q]}$

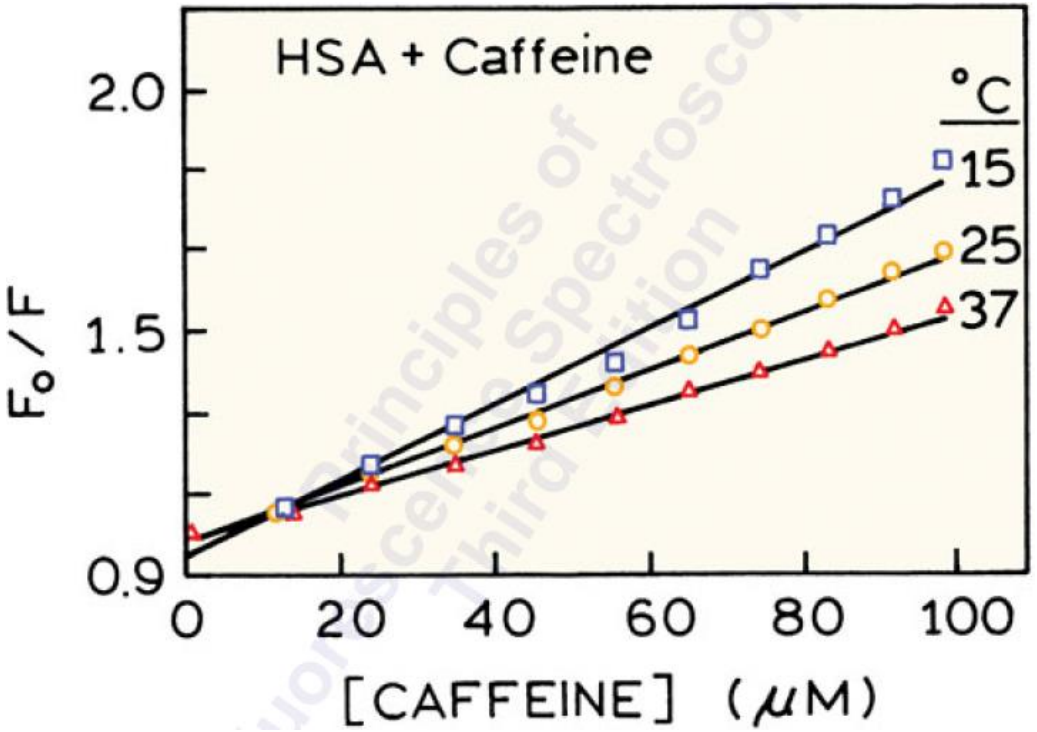
$F_0/F = 1+K_? [Q]$



Pendenza= K_{SV}

* Vedi dopo

Esempio applicativo di Q. statico: studi di interazione



→ In questo caso il Q. è la molecola che vogliamo studiare (la caffeina). Quindi misuriamo la Fluorescenza dell'albumina serica umana (Trp) al variare della concentrazione di caffeina e costruiamo il grafico di SV.

→Il Plot SV è lineare e l'andamento con la temperatura ci dice che il Q. è essenzialmente di tipo statico.

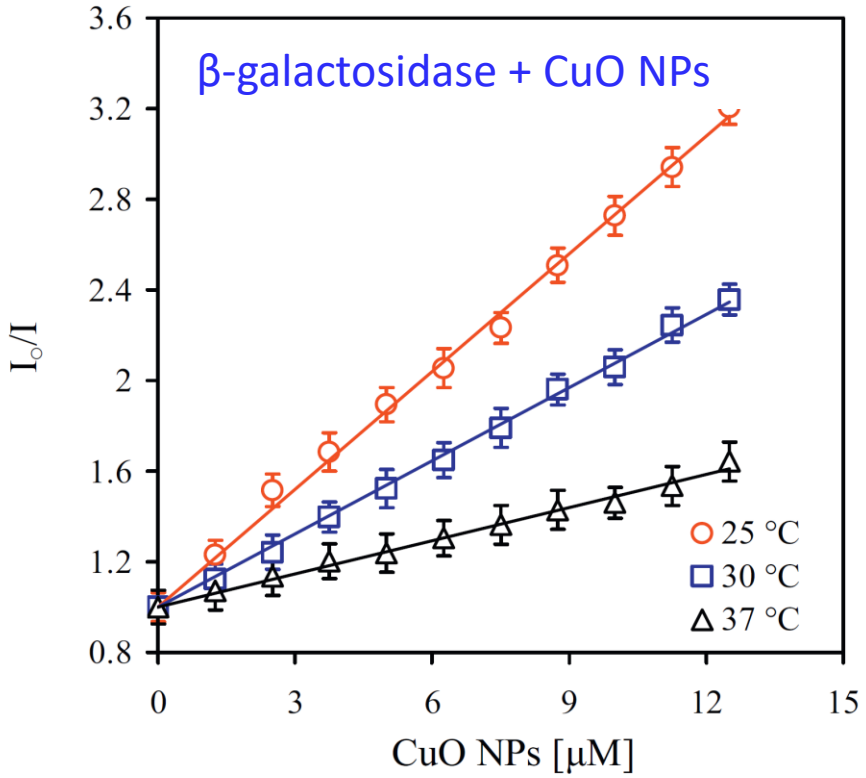
→ Quindi la pendenza della curva è Ks.

$F_0/F = 1 + Ks\,[Q]$

Nota: Vedi anche la parte che segue su fluorescenza e interazioni

Lakowicz, Principles of Fluorescence Spectroscopy; Springer Science+Business Media, LLC

Esempio applicativo di Q. statico: studi di interazione



→ In questo caso il Q. è la nano-particella (NP) di ossido di rame.

→ La fluorescenza è quella intrinseca della β-galactosidase.

→Il Plot SV è lineare e l'andamento con la temperatura ci dice che il Q. è essenzialmente di tipo statico.

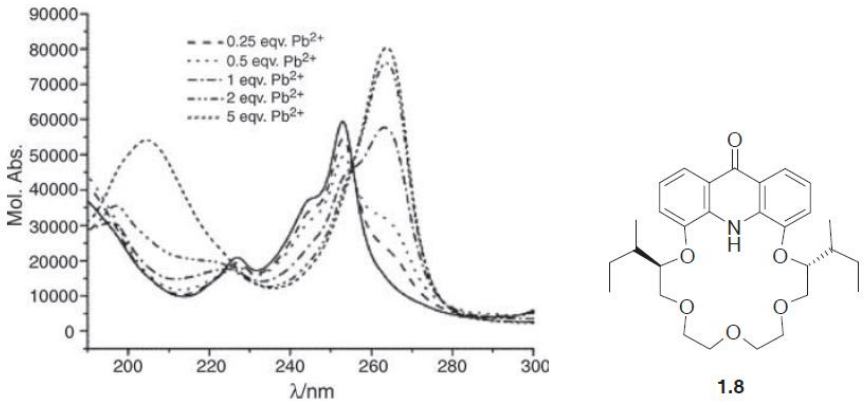
→ Quindi la pendenza della curva è Ks.

Nota: Vedi anche la parte che segue su fluorescenza e interazioni

$F_0/F = 1+Ks\,[Q]$

Stern–Volmer plots for the quenching of β-galactosidase by CuO NPs at three different temperatures.
http://dx.doi.org/10.1016/j.colsurfb.2014.08.035

UV titration



1.8

Figure 1.6 UV-monitored titration of a diisobutyl-substituted acridono-18-crown-6 ligand **1.8** with Pb^{2+} showing an isosbestic point at 271 nm (solid line represents free ligand spectrum, reproduced from [7] with permission from Elsevier).

Unlike fluorescence methods, the observation of one or more clear isosbestic points is common in absorption spectroscopic titrations. An isosbestic point is where the observed absorption intensity is constant throughout the titration. The observation of an isosbestic point is good evidence for the conversion of free host into complex without the involvement of significant intermediate species. Figure 1.6 shows the observed UV-Vis spectra during a titration of a diisobutyl-substituted acridono-18-crown-6 ligand 1.8 with Pb. The isosbestic point occurs at at 271 nm

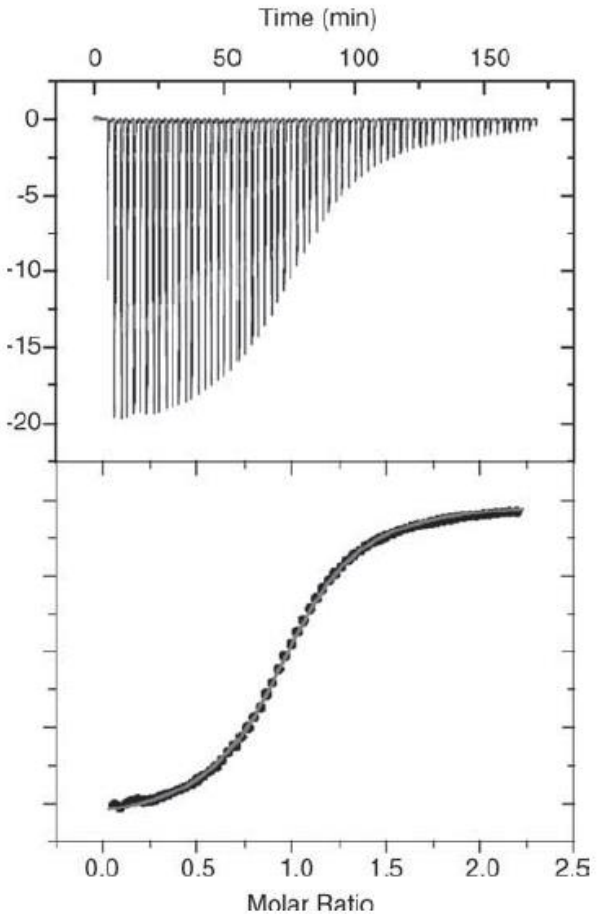
UV-Vis spectroscopic titration (or spectrophotometric titration) involves monitoring the intensity of a electronic absorption band at a particular wavelength that is characteristic of either the complex or free host or guest and is closely analogous to fluorescence titration methods.

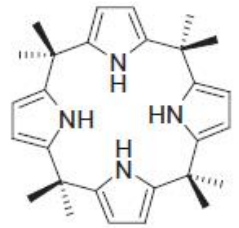
A plot is generated of absorbance intensity vs. concentration of added guest to a solution of constant host concentration. Software can then be used, in conjunction with an appropriate stoichiometry model, to extract the binding constant(s).

Both fluorescent and UV-Vis spectroscopic methods have the advantage over NMR methods that they are more sensitive and hence lower concentrations of host and guest can be used.

Calorimetric Titration

Calorimetric titration, also known as isothermal titration calorimetry (ITC), involves careful measurement of the heat (enthalpy) evolved from a carefully insulated sample as a function of added guest or host concentration. The gradient of the ITC curve can be fitted to determine the binding constant and hence ΔG complex.
Integration of the total area under the ITC plot gives the complexation enthalpy (ΔHcomplex) and hence the technique can give a measurement of all thermodynamic parameters of the system since ΔGcomplex = ΔHcomplex – TΔScomplex.
ITC is useful for determination of binding constants that range from ca.102 – 107 M-1.





1.9

Figure 1.7 ITC data at 25 °C for the binding of $NBu_4^+Cl^-$ by **1.9** in nitromethane – the top plot represents the raw data with the calorimetric response in µcal s^{-1} for each addition of $NBu_4^+Cl^-$ while the lower plot is the titration isotherm fitted to a 1:1 model with kcal per mol $NBu_4^+Cl^-$ added *vs.* mole ratio of $NBu_4^+Cl^-$ to **1.9**. (Reproduced with permission from [8] © 2006, American Chemical Society).