9th International Electronic Conference on Synthetic Organic Chemistry. ECSOC-9
1-30 November 2005. http://www.usc.es/congresos/ecsoc/9/ECSOC9.HTM & http://www.mdpi.net/ecsoc/

[F006]

Fluorescence Correlation Spectroscopy as a Tool for the Study of the Dynamics of Supramolecular and Organized Systems

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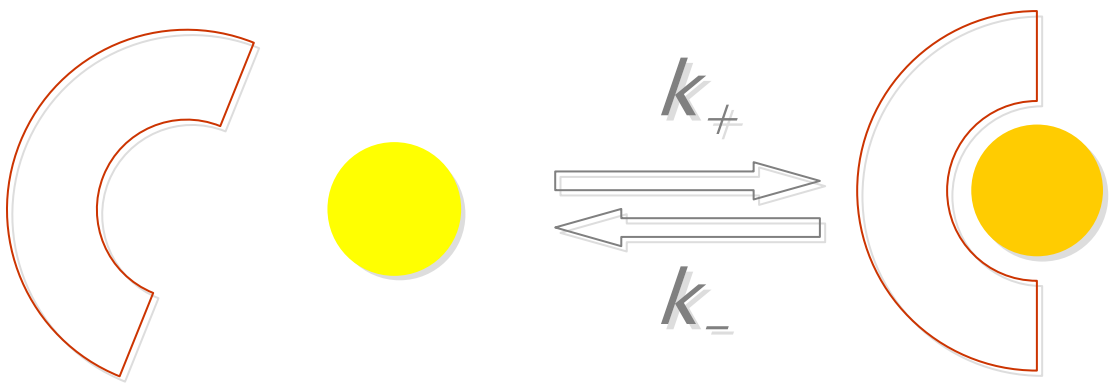
Dynamics of supramolecular systems

Supramolecular system: aggregates stabilized by *non-covalent* bonds



Structure ✓

Stability ✓

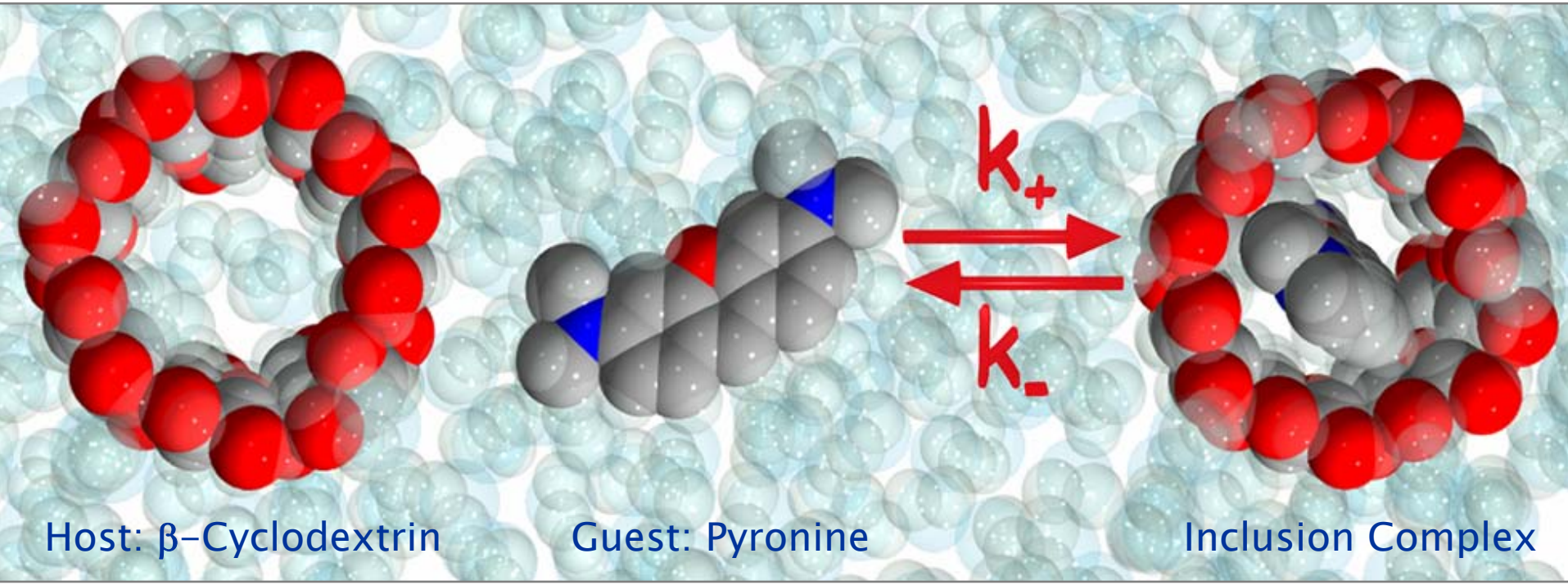
? Dynamics ?

Understanding supramolecular *dynamics* is fundamental for the design and control of functional supramolecular systems. Information on the dynamics complements thermodynamic and structural studies, but cannot be derived from these [1,2,3].

Our objectives:

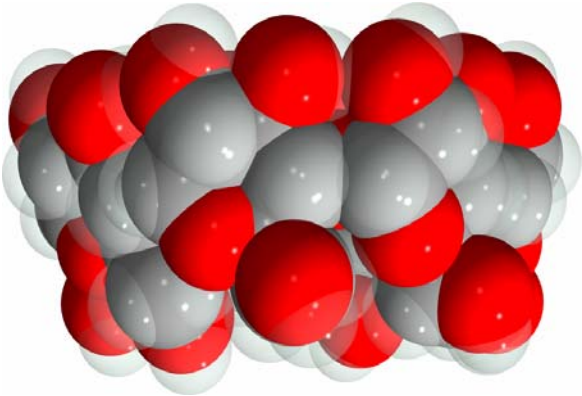
- To adapt the technique of *Fluorescence Correlation Spectroscopy* (FCS) to the study of the *dynamics of association and dissociation* of supramolecular systems and of their diffusional dynamics.
- To understand the underlying kinetic mechanism.

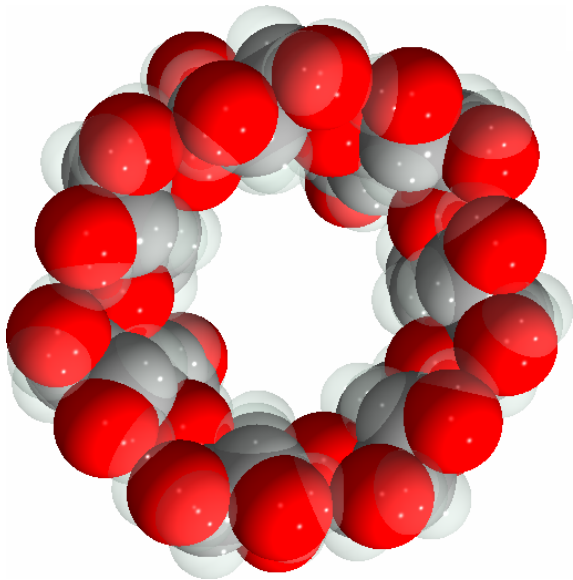
Supramolecular model system

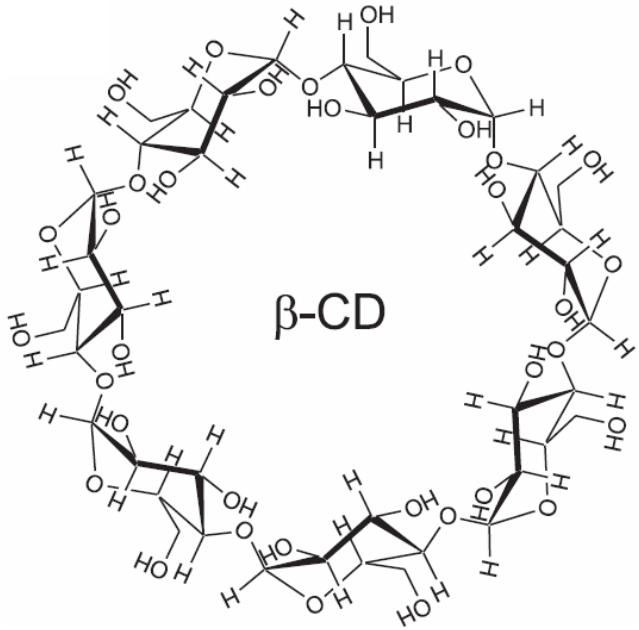


- The inclusion changes the physicochemical properties of the guests [4].
- Cyclodextrines are used as container for functional guests in applications such as protection, controlled release, "targeting", … [5]
- This type of systems represents also a model for the study of ligand-receptor interactions.

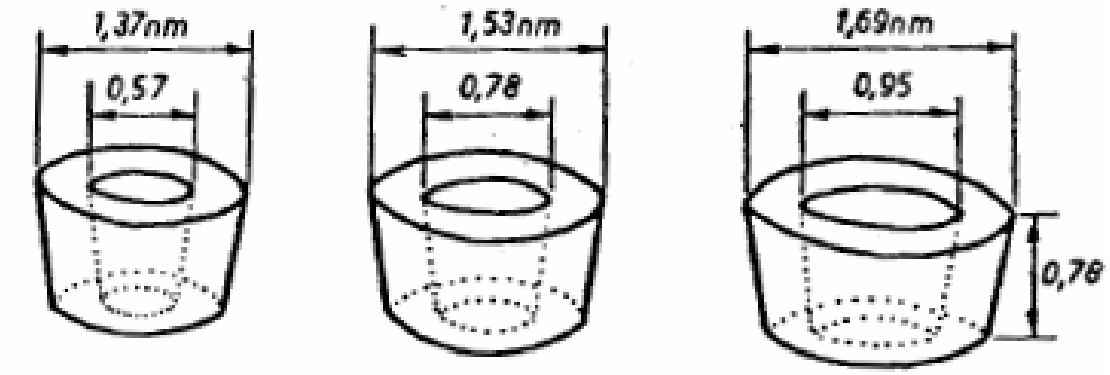
Host: Cyclodextrins



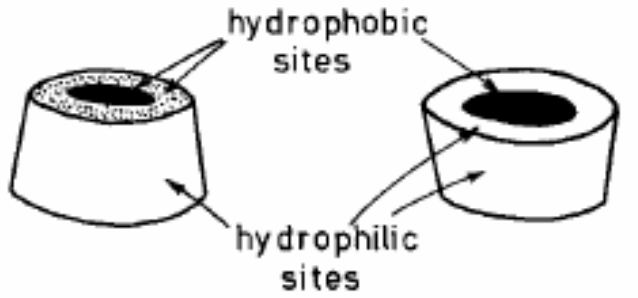




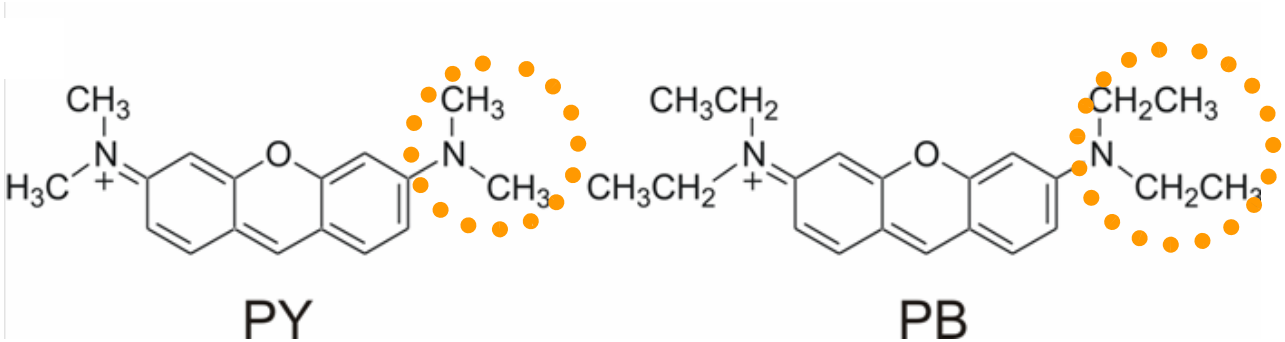
Natural CD's:

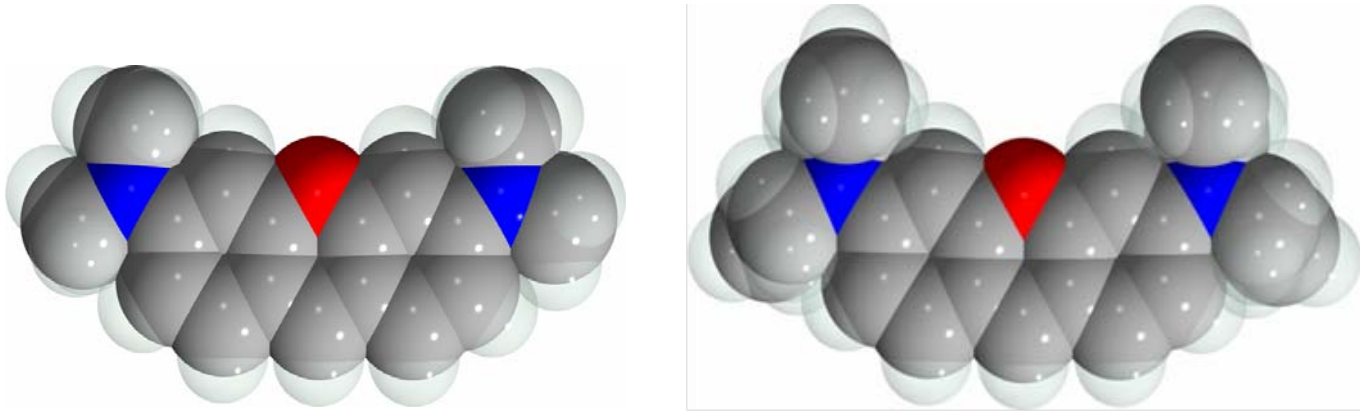


α-CD β-CD γ-CD



Guests: Pyronines Y and B





Pyronine Y

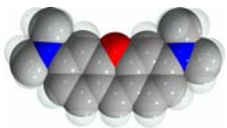
Pyronine B

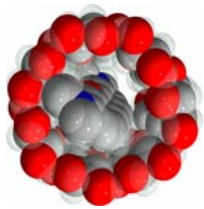
- Cytotoxic dyes used in the Unna Pappenheim stain for ribonucleic acid.
- Simplified models of the xanthene skeleton of rhodamines.

Results from bulk measurements:

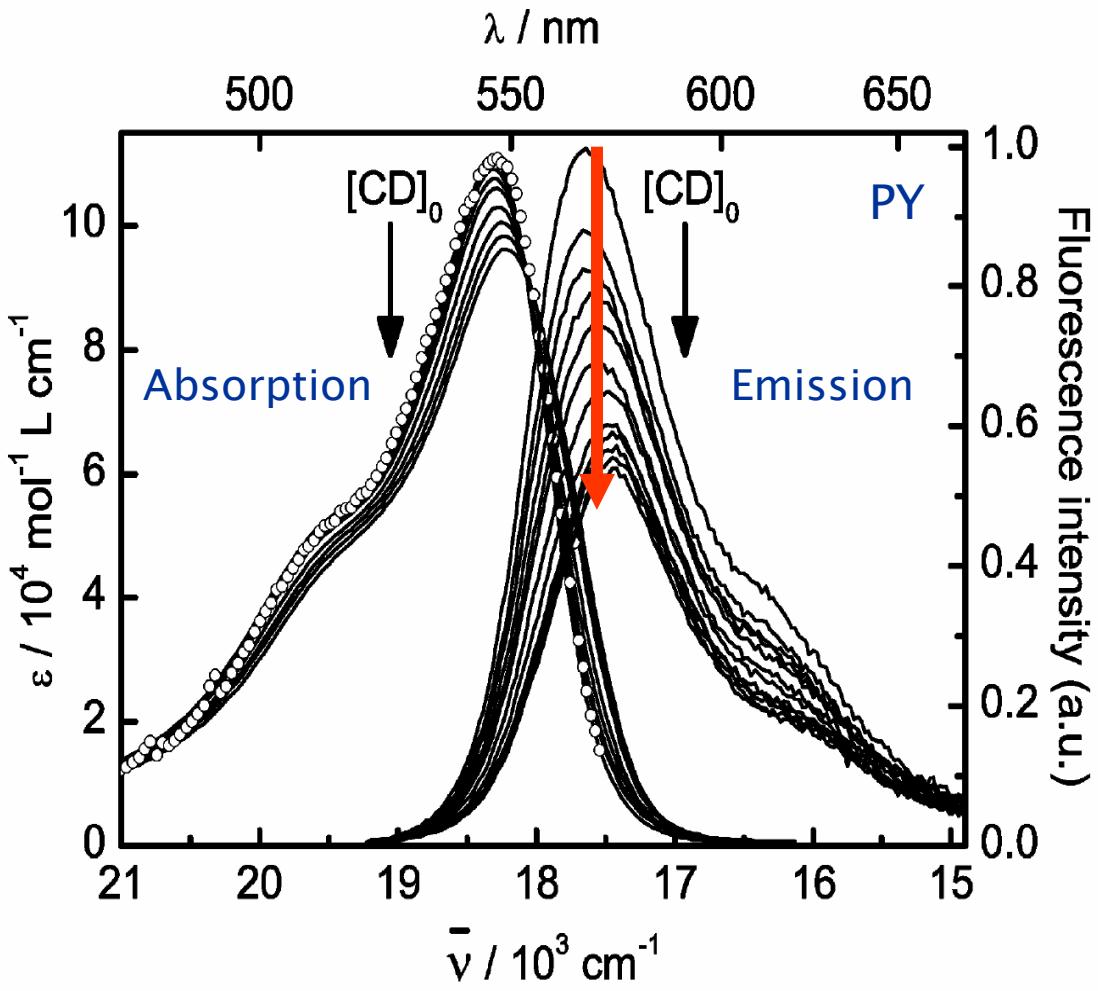
Bulk absorption and emission spectra of the pyronines in the presence of β-CD show the formation of 1:1 inclusion complexes, with a decrease of the fluorescence quantum yield and of the lifetime [6]

$$P + CD \underset{}{\overset{K}{\rightleftharpoons}} P{:}CD$$

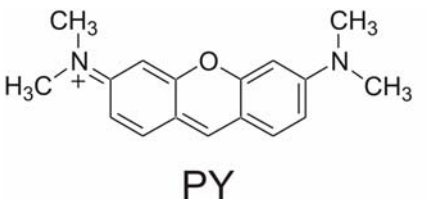




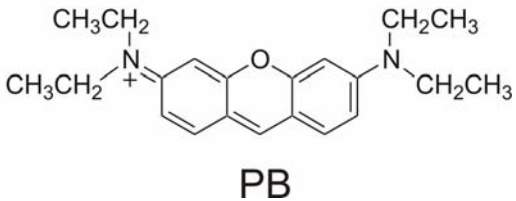
K : association equilibrium constant



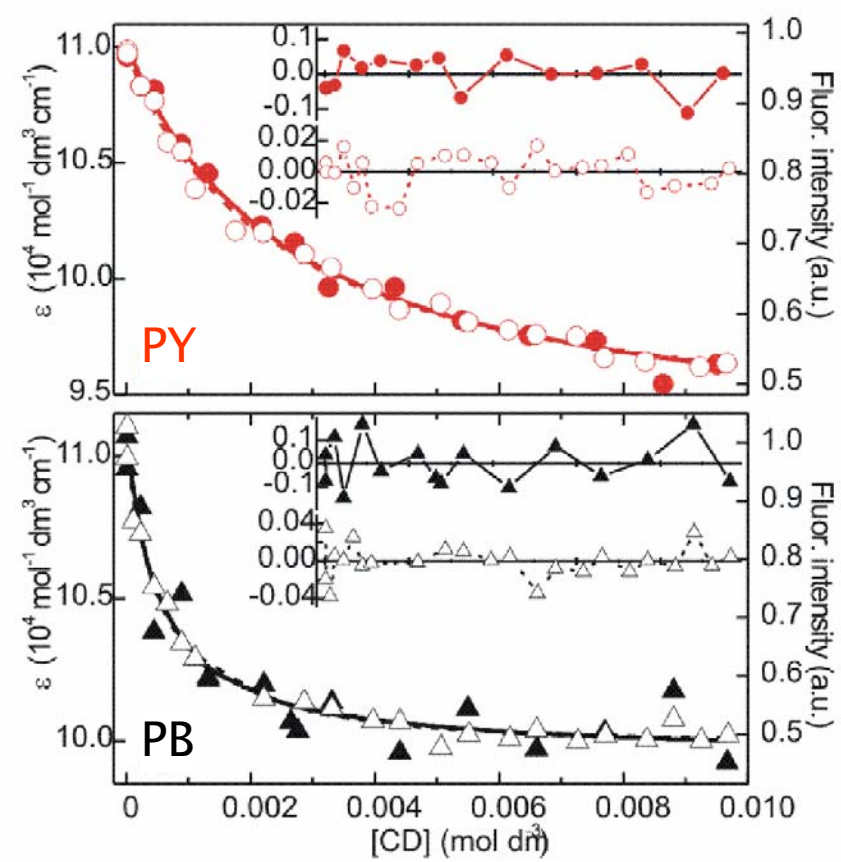
Results from bulk measurements:



PY



PB

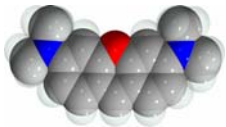


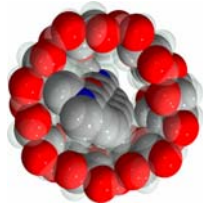
Results of the different titrations for the association equilibrium constant, *K* [6]:

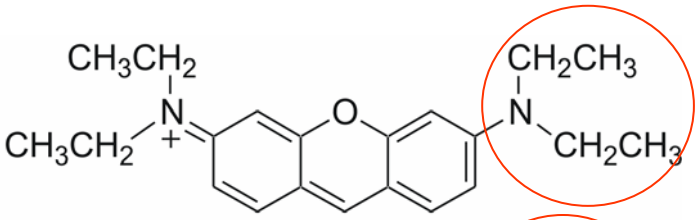
$K/10^3$ mol^{-1} dm^3	PY	PB
absorption spectra	0.39 ± 0.05	2.0 ± 0.4
emission spectra	0.40 ± 0.04	2.1 ± 0.2
fluorescence decays	0.36 ± 0.03	2.0 ± 0.1

Results from bulk measurements:

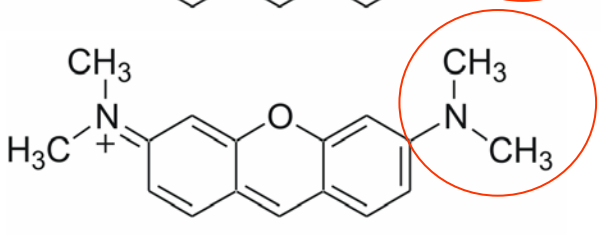
$$P + CD \underset{}{\overset{K}{\rightleftharpoons}} P{:}CD$$







PB: K=2000 M^{-1}



PY: K=400 M^{-1}

The much higher value of the association equilibrium constant K for PB is a surprising result since it is presumed that the more bulky PB would show stronger steric hindrance than PY to enter into the cavity.

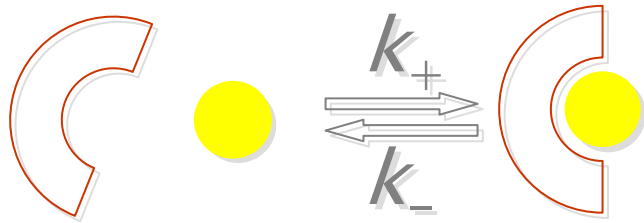
Results from bulk measurements:

What is the reason for this great difference ?

$$K(\mathrm{PB}) \gg K(\mathrm{PY})?$$

In order to understand this the *dynamics* of the inclusion process have to be resolved.

$$K = \frac{k_+}{k_-}$$



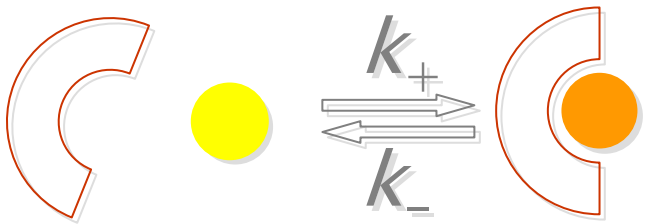
Questions to clarify:

- Do *steric effects* play an important role during the association process ?
- Do *specific interactions* stabilize the complex once formed ?

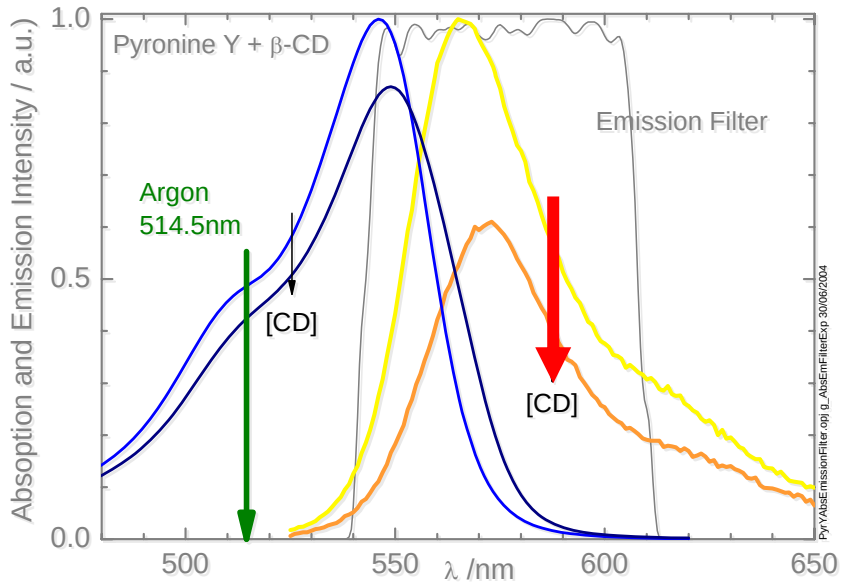
How to observe the dynamic processes:

The inclusion induces observable changes in physicochemical properties:

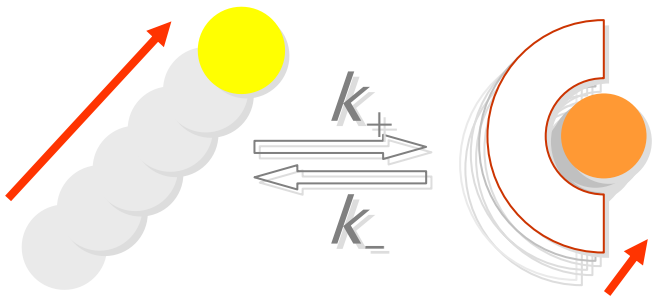
Change in brightness Q



$$Q(P) \approx 2 \cdot Q(P{:}CD)$$

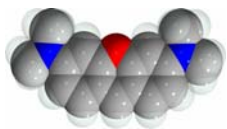


Change in diffusion coefficient D



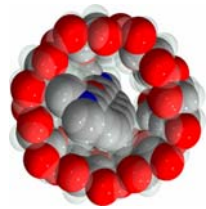
$$D(P) \gg D(P{:}CD)$$

PY



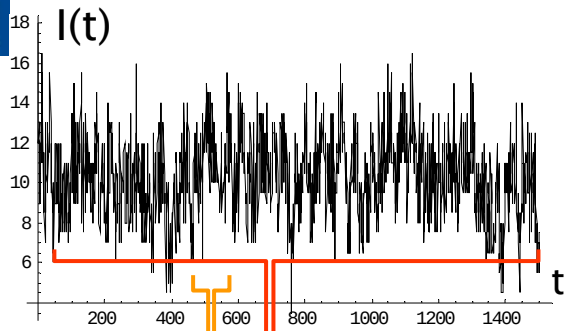
M_W=267 Da

PY:CD

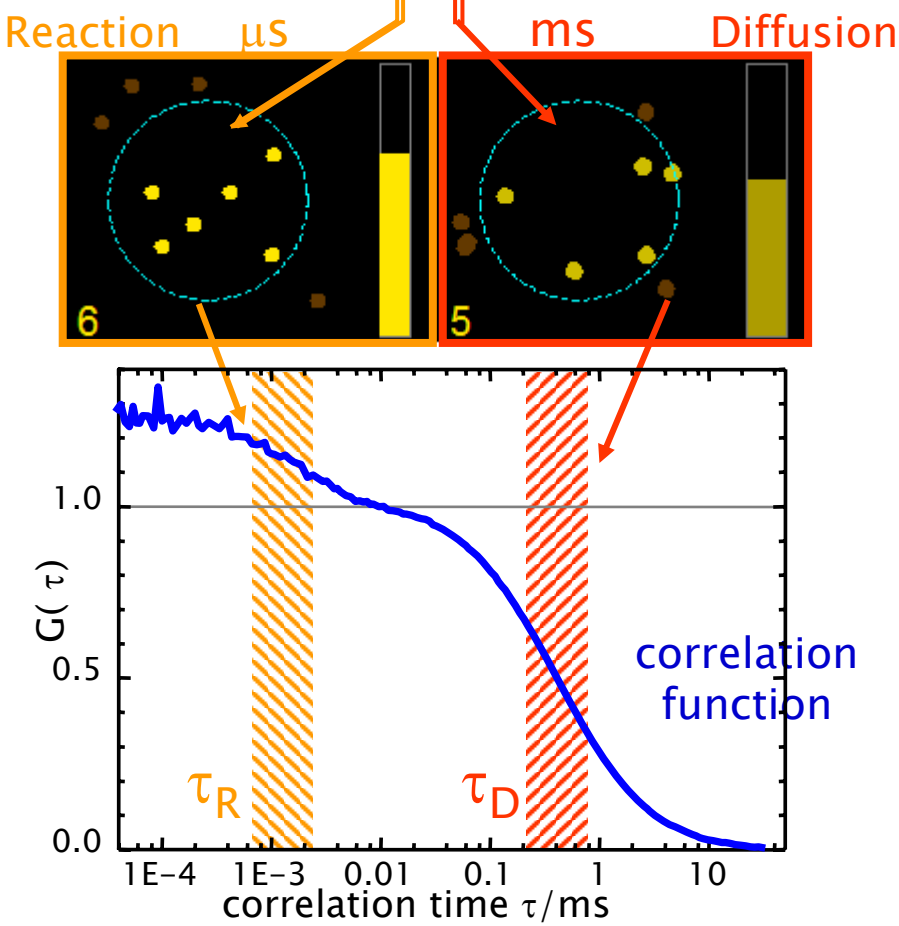


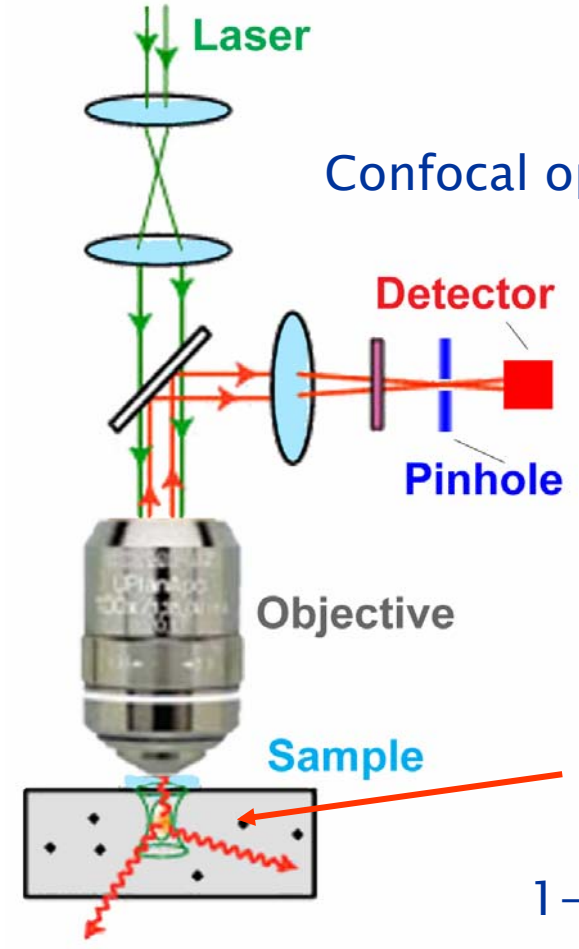
M_W=1402 Da

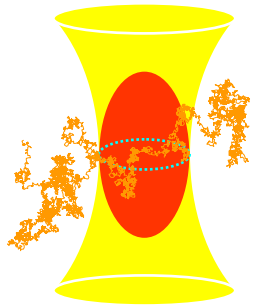
Fluorescence Correlation Spectroscopy (FCS)



FCS: analyses spontaneous fluctuations in fluorescence intensity provoked by molecular processes of systems at thermodynamic equilibrium [7,8].







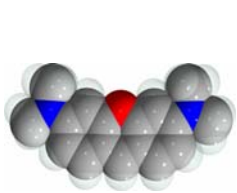
V ≈ 1 fL

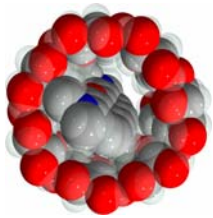
C ≈ 1 nM

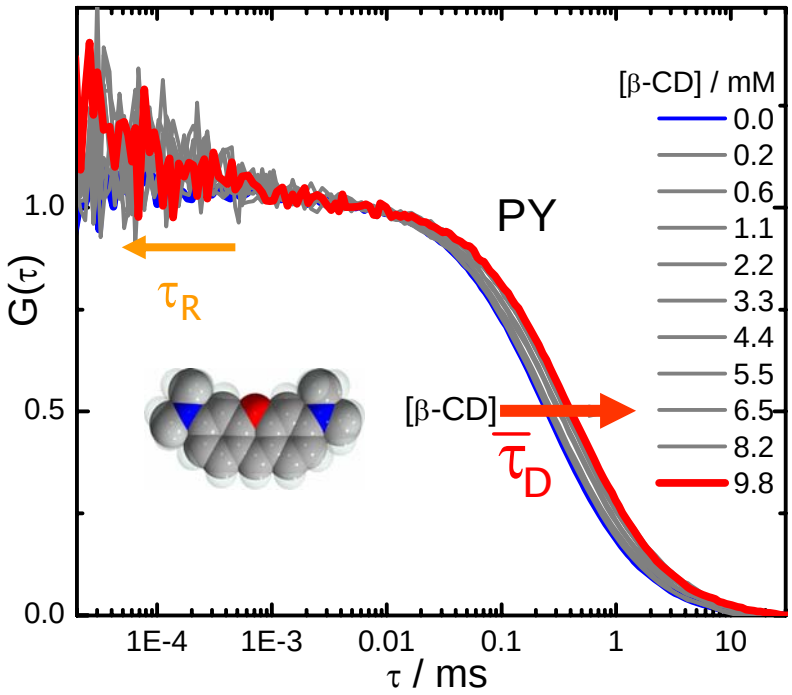
1–10 molecules

FCS: Correlation function

$$P \underset{k_-}{\overset{k_+[CD]}{\rightleftharpoons}} P:CD$$







The addition of CD causes an increase of the observed diffusion time of the pyronine and the appearance of a new reaction term at τ_R which depends on the CD concentration:

Diffusion:

$$\overline{\tau}_D = \frac{w_{xy}^2}{4\bar{D}} \qquad \bar{D} = X_P D_P + X_{PCD} D_{PCD}$$

$\overline{\tau}_D$ observed mean diffusion time
D: diffusion coefficient
w_{xy}: focal radius
X_P, X_{PCD}: molar fractions

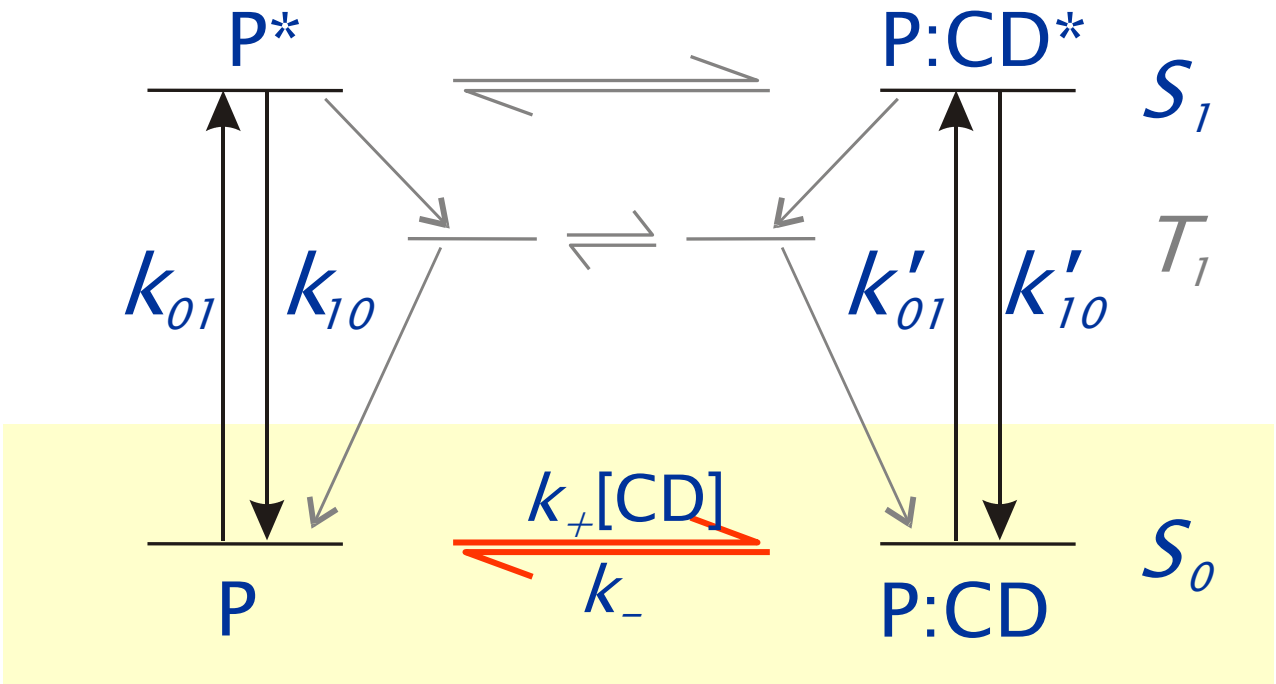
Association / Dissociation:

$$\tau_R = (k_+[CD] + k_-)^{-1}$$

τ_R: "reaction" time

Mechanism: Ground state reaction

The reaction term corresponds to the association/dissociation processes in the ground state since the lifetimes of the molecules are very short and the triplet quantum yield is small:



$$k_+[\mathrm{CD}] \ll k_{01} + k_{10} \quad k_- \ll k'_{01} + k'_{10}$$

FCS: Correlation function for chemical reaction

The change in brightness of two molecules A (P) and B (P:CD) leads to a new "**reaction term**" in the correlation curve:

Under Gaussian illumination and fast exchange: $\tau_R \ll \tau_A, \tau_B$

$$G(\tau) = \frac{1}{N_A + N_B} \underbrace{\left(1 + \frac{\tau}{\overline{\tau}_D}\right)^{-1} \left(1 + \frac{\tau}{(w_z/w_{xy})^2 \overline{\tau}_D}\right)^{-\frac{1}{2}}}_{\text{Diffusion with mean diffusion time}} \underbrace{\left(1 + A_R e^{-\tau/\tau_R}\right)}_{\text{Reaction}}$$

Relaxation time: $\tau_R = \dfrac{1}{k_+[\mathrm{CD}]_0 + k_-}$ Amplitude: $A_R = \dfrac{(Q_A - Q_B)^2 N_A N_B}{(Q_A N_A + Q_B N_B)^2}$

Limiting values: $G(\tau \to 0) = \dfrac{N_A Q_A^2 + N_B Q_B^2}{(Q_A N_A + Q_B N_B)^2}$ $G(\tau \to \infty) = 0$

w_{xy}, w_z : focal radii. N_A and N_B: mean numbers of free guest and complex in the sample volume.
Q_A, Q_B: Brightness of A and B.

FCS: Correlation function for chemical reaction

Full correlation function: two additional terms, the formation of triplet and the antibunching term, are multiplied to the equation deduced before:

$$G(\tau) = 1 + \frac{1}{\overline{N}} \underbrace{\left(1 + \frac{\tau}{\overline{\tau}_D}\right)^{-1} \left(1 + \frac{\tau}{(\frac{w_z}{w_{xy}})^2 \overline{\tau}_D}\right)^{-\frac{1}{2}}}_{\text{Diffusion}} \underbrace{\left(1 + A_T e^{-\tau/\tau_T}\right)}_{\text{Triplet}} \underbrace{\left(1 + A_R e^{-\tau/\tau_R}\right)}_{\text{Reaction}} \underbrace{\left(1 - A_F e^{-\tau/\tau_F}\right)}_{\text{Antibunching}}$$

Global "Target" Analysis: recalculates in each iteration of the global fit the individual parameters τ_D, A_R, and τ_R as function of the equilibrium constant K and $[CD]_0$ and of the global parameters τ_A, τ_B, k_- and Q.

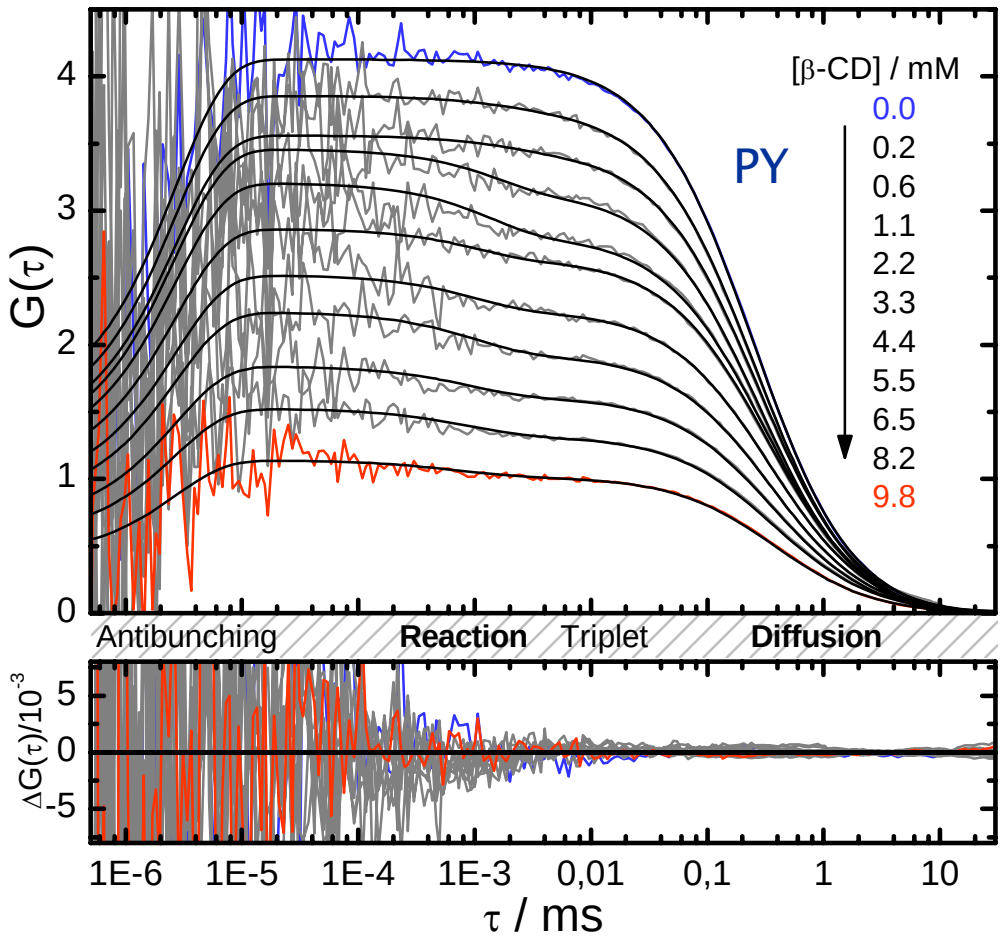
$$\overline{\tau}_D = \frac{\tau_A \left(1 + K[CD]_0\right)}{1 + \frac{\tau_A}{\tau_B} K[CD]_0} \qquad A_R = \frac{K[CD]_0 (1-Q)^2}{(1 + QK[CD]_0)^2} \qquad \tau_R = \frac{1}{k_-(1 + K[CD]_0)}$$

$$Q = Q_B / Q_A$$

FCS: Global Target Analysis

Global "Target" Analysis of series of correlation curves yields values for the individual dynamic parameters [9]:

Fluorescence Correlation Curves



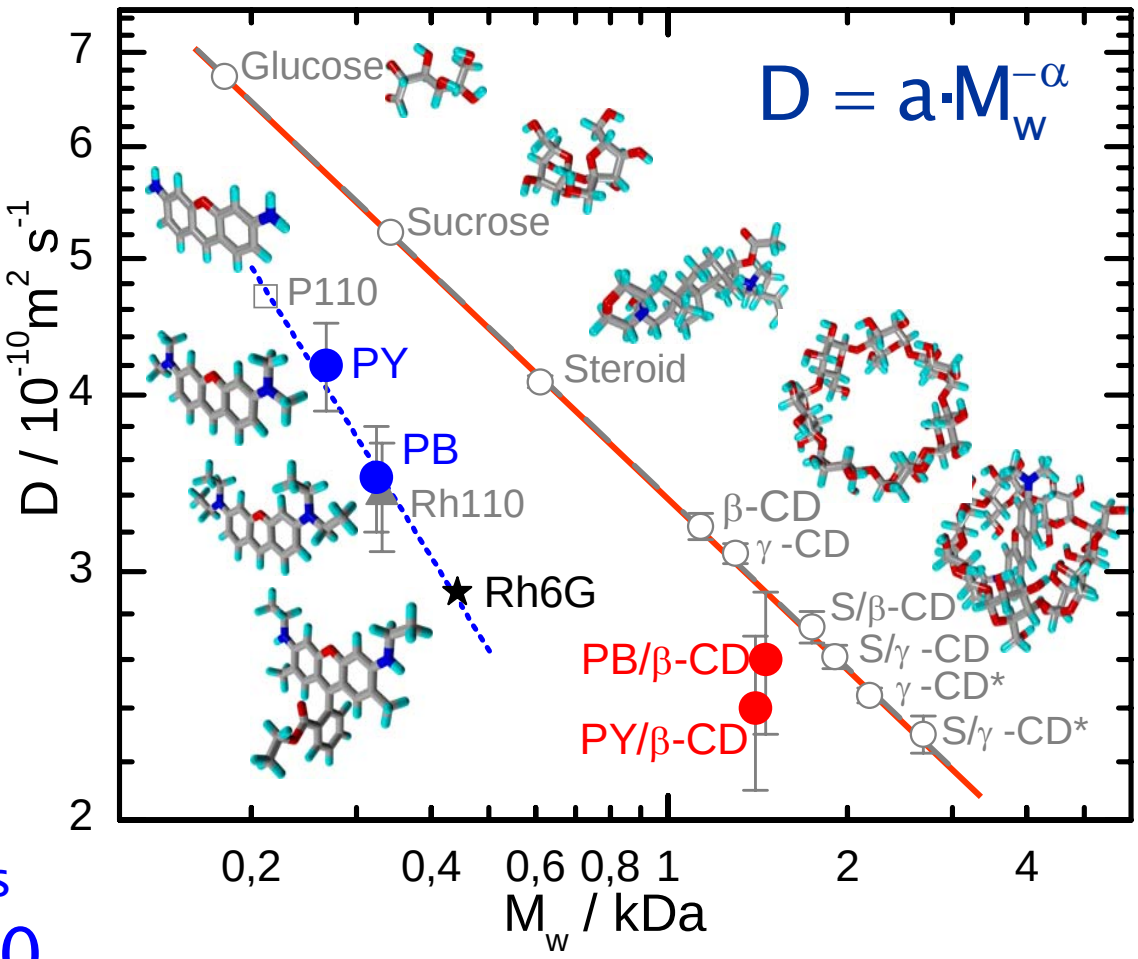
	PY	PB
Diffusion times		
τ_P/ms	0.25 ± 0.2	0.30 ± 0.02
τ_{PCD}/ms	0.45 ± 0.06	0.40 ± 0.06
Rate constants		
$k_+/10^9\ M^{-1}s^{-1}$	0.2 ± 0.1	0.15 ± 0.05
$k_-/10^4\ s^{-1}$	50 ± 30	7.6 ± 2.7

- The diffusion times of the pyronines are similar but much lower than those of the complexes.
- The association rate constants k_+ of PB and PB are equal, whereas the dissociation rate constants k_- are different and account for the different stabilites of the complexes

Diffusion Coefficients

The diffusion times can be converted to diffusion coefficients and compared to known values of similar molecules:

	D 10^{-10} m^2s^{-1}	M_W Da
PY	4.2 ± 0.3	267
PB	3.5 ± 0.3	324
PYCD	2.4 ± 0.3	1402
PBCD	2.6 ± 0.3	1459



- The P:CD complexes behave like nearly compact spheres.
- The pyronines however show lower diffusion coefficients, probably due to the more planar xanthenic system.

compact spheres $\alpha = 1/3$

Pyronines $\alpha = 0.70$

Mechanistic study of association/dissociation

$$P + CD \underset{k_-}{\overset{k_+}{\rightleftharpoons}} P{:}CD$$

	k_+ / $10^9\ M^{-1}s^{-1}$	k_d / $10^9\ M^{-1}s^{-1}$	k_{-d} / $10^9\ s^{-1}$
PY	0.2 ± 0.1	7.5 ± 0.5	1.7 ± 0.1
PB	0.15 ± 0.05	7.4 ± 0.5	1.3 ± 0.1

k_d: diffusion limited rate constant.

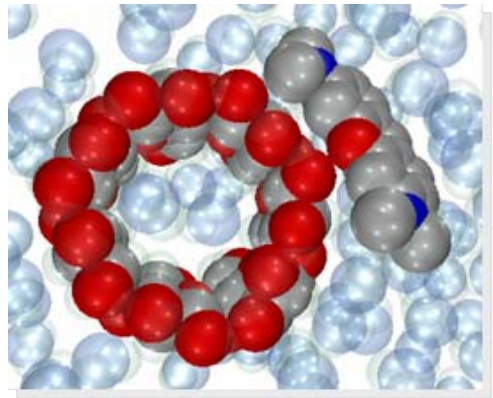
$$k_d = 4\pi D\ R\ N_A$$

$$P + CD \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} P \cdot CD \underset{k_{-r}}{\overset{k_r}{\rightleftharpoons}} P{:}CD$$

Encounter Complex

The estimated diffusion-limited rate constants k_d are much higher than the observed association rate constants k_+, which are obviously limited by other processes than the diffusion itself.

We assume therefore the formation of an *encounter complex* P·CD where P and CD are in the same solvent cage.



$$K = \frac{k_+}{k_-} \approx 5\,M^{-1} \cdot \frac{k_r}{k_{-r}} \qquad (k_{-d})^{-1} = \frac{R^2}{4D} \approx 500\,ps$$

Mechanistic study of association/dissociation

$$P + CD \underset{k_-}{\overset{k_+}{\rightleftharpoons}} P{:}CD$$

	k_+ / $10^9\ M^{-1}s^{-1}$	k_d / $10^9\ M^{-1}s^{-1}$	k_{-d} / $10^9\ s^{-1}$	k_r / $10^9\ s^{-1}$
PY	0.2 ± 0.1	7.5 ± 0.5	1.7 ± 0.1	0.05 ± 0.02
PB	0.15 ± 0.05	7.4 ± 0.5	1.3 ± 0.1	0.03 ± 0.01

$$k_r = \frac{k_+ k_{-d}}{k_d - k_+}$$

$$P + CD \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} P \cdot CD \underset{k_{-r}}{\overset{k_r}{\rightleftharpoons}} P{:}CD$$

Association

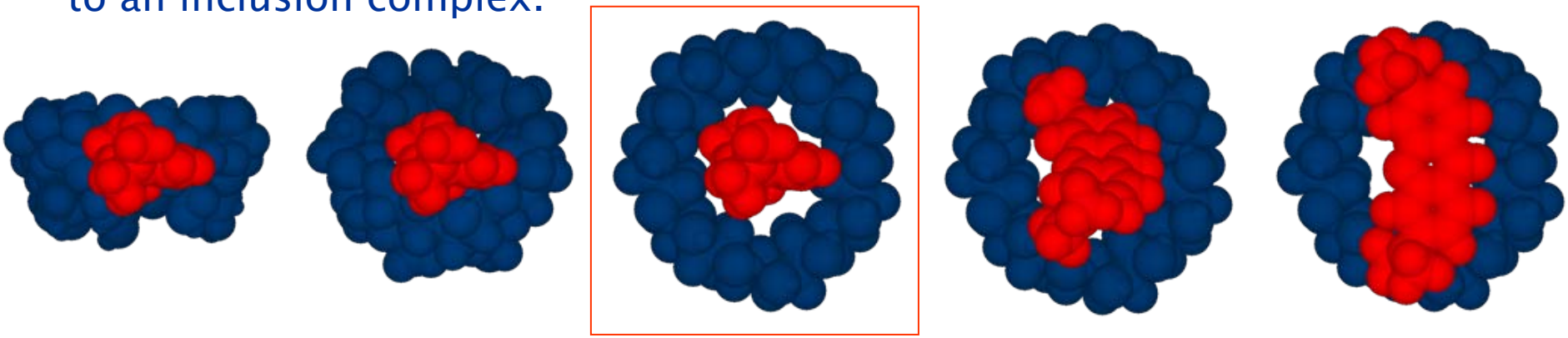
The estimated rate constants for the unimolecular inclusion process are equal for PY and PB.

1) The unimolecular inclusion step limits the total de association rate

2) The different end groups have nearly no influence on this process.

Influence of steric hindrance during inclusion

We suppose in a simplified model that only those collisions in which host and guest are in favorable relative orientations are successful and give rise to an inclusion complex.



- Simulations indicate that the geometrical reorientation may be the rate determining step in the inclusion process and that the rate of inclusion may be dictated by steric effects.
- The remaining *gap* between pyronine and CD is the *critical dimension* which determines the inclusion rate. The different alkyl substituents of PY and PB have only a weak influence on this value.

Mechanistic study of association/dissociation

$$P + CD \underset{k_-}{\overset{k_+}{\rightleftharpoons}} P{:}CD$$

	k_+ / $10^9\ M^{-1}s^{-1}$	k_- / $10^4 s^{-1}$	k_d / $10^9\ M^{-1}s^{-1}$	k_{-d} / $10^9\ s^{-1}$	k_r / $10^9\ s^{-1}$	k_{-r} / $10^4\ s^{-1}$
PY	0.2 ± 0.1	50 ± 30	7.5 ± 0.5	1.7 ± 0.1	0.05 ± 0.02	50 ± 30
PB	0.15 ± 0.05	7.6 ± 2.7	7.4 ± 0.5	1.3 ± 0.1	0.03 ± 0.01	7.6 ± 2.7

$$P + CD \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} P \cdot CD \underset{k_{-r}}{\overset{k_r}{\rightleftharpoons}} P{:}CD$$

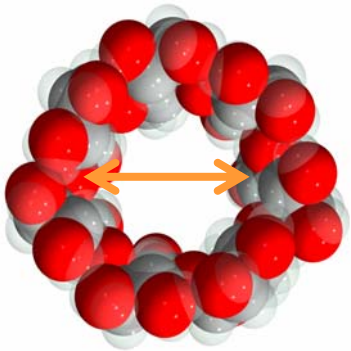
Dissociation

1) The overall dissociation rate constant is limited by the rate constant k_{-r}, with which the guest leaves the host forming again an encounter complex. This is the slowest of all involved steps.

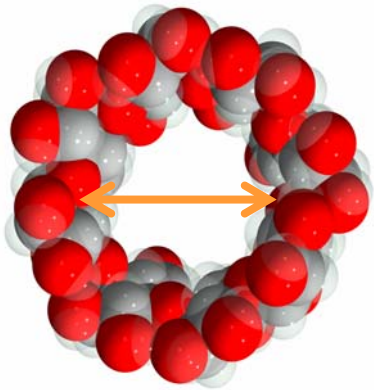
2) Specific interactions between the positively charged xanthene moiety of the pyronines and the electron-rich glucosidic oxygens of the β-CD cavity probably stabilize the inclusion complexes of both pyronines, but seem to be much stronger in the case of PB.

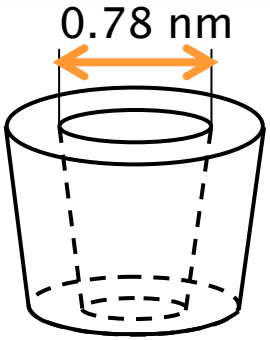
Present studies: Change in cavity size

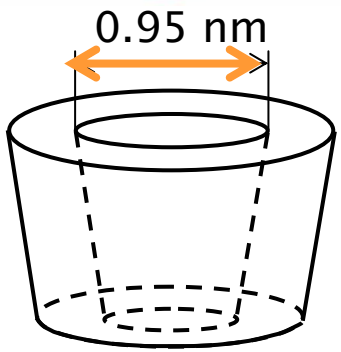
β-CD



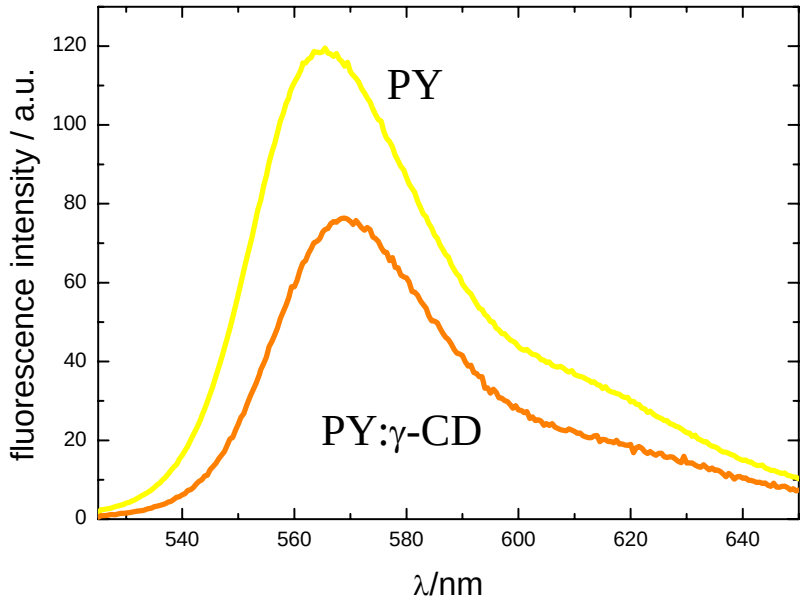
γ-CD







Bulk Fluorescence Study



K/M^{-1}	β-CD	γ-CD
PY	400	40
PB	2000	210

Preliminary results with the wider γ-CD show that:

- A higher association rate constant is observed in accordance to the higher inclusion probability predicted by the simulations.
- A much higher dissociation rate constant seems to be responsible for the much lower stability equilibrium constants as compared to those with β-CD.

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Acknowledgements

Collaborators:

USC
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Wajih Al-Soufi
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Jorge Bordello
Mayra Moure
Arturo Teijeiro

Heinrich-Heine Universität
Claus A. M. Seidel
Suren Felekyan
Ralf Kühnemuth

Financial support:

- *Ministerio de Educación y Ciencia* (CTQ2004-07683-C02)
- *Xunta de Galicia*
- *BMFT* (BioFuture Grant 0311865).