Evidence of cyclodextrin aggregation obtained with Fluorescence Correlation Spectroscopy

W. Al-Soufi, D. Granadero, J. Bordello, S. Freire, L. Piñeiro and M. Novo
Universidade de Santiago de Compostela, Facultade de Ciencias,
Departamento de Química Física. E-27002 Lugo, Spain.

Abstract

We have studied the complexation between an adamantane derivative labelled with the fluorescent probe Alexa 488 and the three natural cyclodextrins (α-, β- and γ-CD) by Fluorescence Correlation Spectroscopy (FCS), demonstrating the ability of this technique to detect association and to determine the corresponding equilibrium constants with no need for changes in the fluorescence properties of the guest. At low CD concentrations the observed increase of the diffusion time of the probe is mainly due to the complexation of the adamantyl moiety, but further changes are observed when increasing CD concentration that are attributed to the formation of CD aggregates. These aggregates appear at quite low CD concentrations and seem to be formed by a small number of CD molecules. These results show the potential of FCS for the study of CD self-assembly, a recently-recognized phenomenon that could be used to improve certain applications of CDs.

Introduction

The study of supramolecular systems requires a detailed characterization regarding stoichiometry, affinity, structure, heterogeneity and dynamics. To gain insight into these aspects is in general very challenging and requires the combination of various techniques, such as calorimetry, solubility experiments, NMR, light scattering, microscopy and absorption and fluorescence spectroscopies. However, Single-Molecule Fluorescence Detection (SMFD) techniques, which are based on the detection of the individual spectral and temporal properties of single labelled molecules, can be used to obtain most of the mentioned information with great advantages against ensemble measurements.[1-5] Previous studies by our group have shown the potential of one of these techniques, Fluorescence Correlation Spectroscopy (FCS), to obtain thermodynamic and dynamic information on host-guest supramolecular association.[6-10]

Cyclodextrins (CDs) are water-soluble toroidally shaped polysaccharides with a highly hydrophobic central cavity that can host a variety of organic and inorganic substrates.[11, 12] The ability to form inclusion complexes is the basis for their widespread applications, such as their use in pharmaceutical technology to enhance the solubility of lipophilic water-insoluble drugs and in drug delivery for controlled release.[13-18] The three native cyclodextrins, called α-, β- and γ-CD, have increasing cavity sizes (see Figure 1) and can therefore form complexes of different stability and stoichiometry with a certain substrate. The thermodynamic properties of CD complexes can only be understood on the basis of dynamic information, which can be obtained using FCS.[6, 8]

Although some unexplainable phenomena (such as opalescence) were observed in CD solutions for many decades, only recently CD self-assembly was recognized.[19, 20] Addition of

hydrogen-bond-disrupting agents or increase of the pH were proposed to inhibit the turbidity and to enhance the solubility of the native CDs in aqueous solution. In the last decade, the aggregates of the three native CDs have been characterized using static and dynamic light scattering (SLS and DLS).[21-24] Recently it has been shown that CD-drug inclusion complexes also aggregate and that the drug's nature has a great influence on the size and structure of CD aggregates and the aggregation dynamics.[25-29] It must be noted that, if controlled, the self-assembly of CDs may offer new possibilities in different applications of CDs. Therefore, further studies are necessary to characterize CD aggregates and to elucidate the influence of the included compounds and the physicochemical environment on their formation. FCS and other SMFD techniques are found to be very suitable for this purpose.

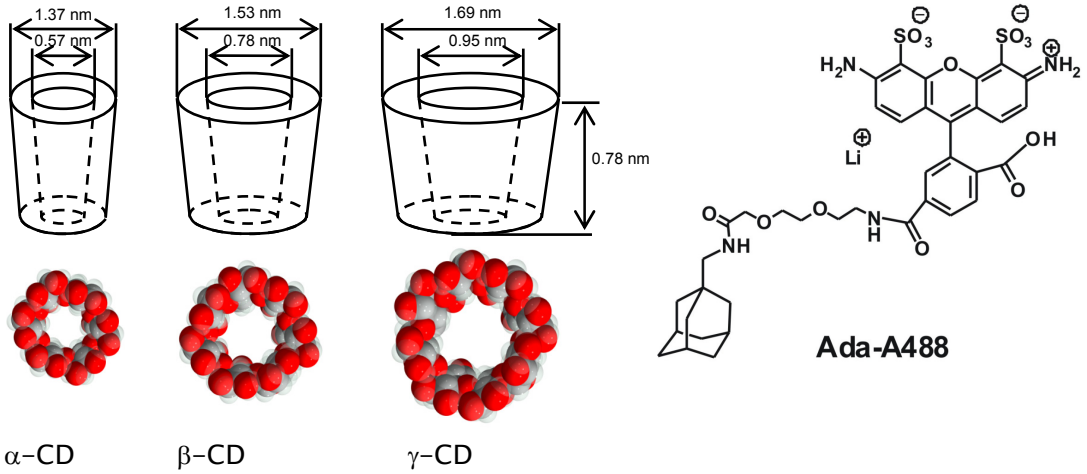

Figure 1. Left: molecular structures of the three natural cyclodextrins and schematic representation of their dimensions. Right: molecular structure of the adamantane derivative labelled with the fluorescent probe Alexa 488 used as guest in this study.

Theory

FCS analyzes the fluorescence intensity fluctuations that are caused by the spontaneous variations in the number of fluorescent molecules in the confocal sample volume due to translational diffusion. The observed fluorescence intensity fluctuates at a time scale given by the mean residence time of a fluorophore in the sample volume. The intensity fluctuations are analyzed by the temporal autocorrelation function G(τ) as function of the correlation time τ.

In this work we focus on the time dependent part of the correlation function describing pure translational diffusion of a single fluorescent species in and out of a sample volume $G_D(\tau)$:

$$G_D(\tau) = \frac{1}{N}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\left(1+\left(\frac{w_{xy}}{w_z}\right)^2\frac{\tau}{\tau_D}\right)^{-\frac{1}{2}} \qquad (1)$$

A three-dimensional Gaussian sample volume is assumed with radial and axial $1/e^2$ radii w_{xy} and w_z, respectively, and a translational diffusion (transit) time of the molecules across the sample volume τ_D, which is related to the translational diffusion coefficient D as follows: $D = w_{xy}^2/4\tau_D$. The radius of the sampling volume, w_{xy}, is determined from a calibration

measurement with a reference dye with known diffusion coefficient as described in the experimental section. Also the mean number N of fluorescent molecules within the sample volume is obtained.

In the case of a fluorescent guest that binds to a non-fluorescent host and under the condition that the exchange of the fluorophore between free and bound states is much faster than the typical transit time of the fluorophore across the sample volume, the two states of the fluorophore (free and bound) will not be seen by FCS as two distinct species, but as a single one with a mean diffusion time $\overline{\tau}_D$. The value of $\overline{\tau}_D$ depends then on the individual diffusion coefficients D_f and D_b of free and bound fluorophore and on the molar fractions of these species. In the case of a 1:1 complexation and assuming that the free host concentration is always much higher than that of the guest, the mean diffusion time $\overline{\tau}_D$ can be expressed as function of the total host concentration $[H]_0$, the association equilibrium constant K and the limiting values of the diffusion times of free and bound dye, τ_f and τ_b, respectively:

$$\overline{\tau}_D = \frac{\tau_f(1+K[H]_0)}{1+\frac{\tau_f}{\tau_b}K[H]_0} \tag{2}$$

For a fluorophore that follows 1:1 and 1:2 complexation processes with association equilibrium constants K_1 and K_2, the mean diffusion time $\overline{\tau}_D$ is then given by:

$$\overline{\tau}_D = \frac{1+K_1[H]_0+K_1K_2[H]_0^2}{\tau_f^{-1}+\tau_{b1}^{-1}K_1[H]_0+\tau_{b2}^{-1}K_1K_2[H]_0^2} \tag{3}$$

Results and discussion

Fluorescence correlation curves of Ada-A488 in the presence of increasing concentrations of each type of CDs were obtained from FCS measurements. Figure 2 shows those obtained for β-CD, where it can be seen that the correlation time shifts towards longer times as the concentration of CD is increased. This shows that there is an interaction between the adamantane derivative labelled with the fluorescent probe Alexa 488 and β-CD. The same behaviour is observed with the other two CDs, although it is necessary to achieve much higher CD concentrations in order to see variations in the correlation time. This shows that the interaction of the adamantane derivative is much stronger with the β-CD than with the other two CDs.

Once the experimental fluorescence correlation curves are obtained, equation (1) is fitted to them to obtain the diffusion correlation times. Figures 3, 4 and 5 show the experimental values of τ_D as function of CD concentration for β-CD, α-CD and γ-CD, respectively.

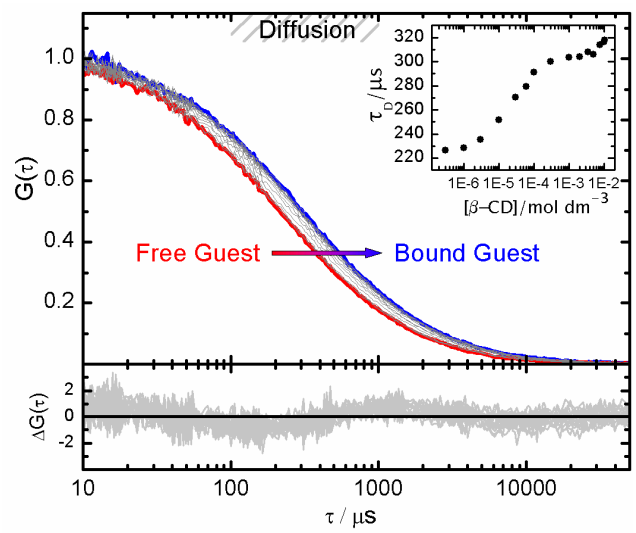

Figure 2. Upper panel: Normalized experimental fluorescence intensity correlation curves G(τ) of Ada-A488 in the presence of increasing concentrations of β-CD from [β-CD]=0 (red curve) to [β-CD]= 10×10^{-3} mol dm^{-3} (blue curve). Inset: logarithmic plot of the diffusion time τ_D versus β-CD concentration. Lower panel: Normalized residuals of G(τ).

At low CD concentrations (less than 2 mM), the interaction between Ada-A488 and β-CD can be explained by a 1:1 complexation equilibrium (equation (2)) and a value for K is obtained which is in good agreement with that reported for adamantane.[10] Nevertheless, when the concentration of β-CD is further increased the diffusion time increases systematically in spite of the fact that all adamantane molecules are already in their complexed form. The whole range of experimental diffusion times can be explained by a 1:1+1:2 complexation equilibrium model (equation (3)). From the analysis of this data, no elucidation of the structure of the 1:2 complex is possible since it can be either a complex in which the adamantane moiety is surrounded by two molecules of cyclodextrin or the adamantane moiety interacts with an aggregate of two cyclodextrins. Experiments with a fluorescence-labelled β-CD are being performed in order to clear up this point.

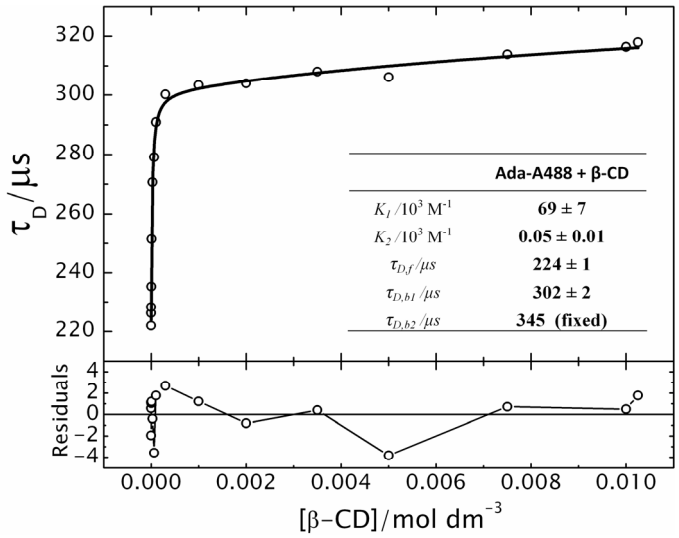

Figure 3. Upper panel: Mean diffusion times τ_D as function of β-CD concentration determined from individual fits of the autocorrelation function to the normalized experimental correlation curves of Figure 2. The black line represents the fitted curve of a 1:1+1:2 complexation model to τ_D. Parameters resulting from the fit are given in the table inserted. Lower panel: Residuals of the fit.

For α-CD and γ-CD, we find that the interaction with the adamantane derivative can be explained by a 1:1 complexation mechanism only within the range of very low CD concentrations, as shown in Figures 4 and 5. A further increase of the diffusion time is observed as the CD concentration is increased which is much stronger than in the case of β-CD and suggests the appearance of CD aggregates. The high values of the obtained diffusion times indicate that the aggregates are formed by assembly of several CD molecules and a mixture of aggregates of different sizes may be present in the solutions at high concentrations.

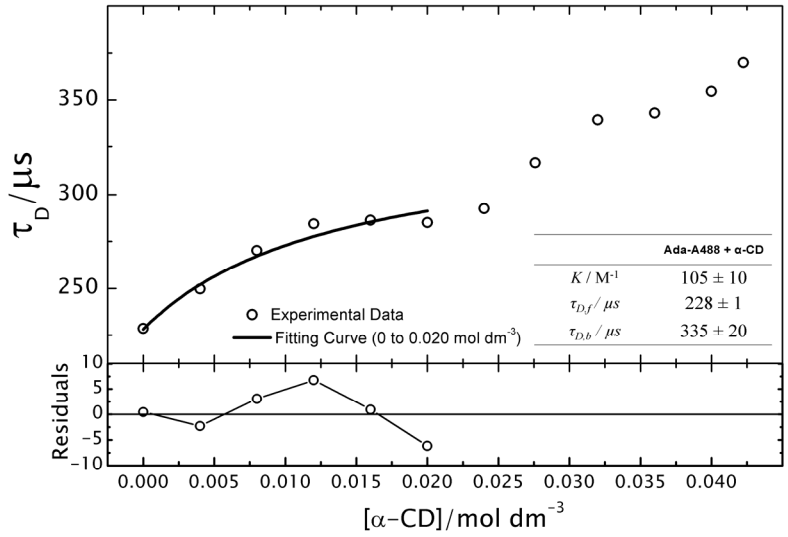

Figure 4. Upper panel: Mean diffusion times τ_D as function of α-CD concentration determined from individual fits of autocorrelation function to the normalized experimental correlation curves measured for α-CD. The black line represents the fitted curve of a 1:1 complexation model to τ_D up to 0.020 mol dm^{-3}. Parameters resulting from the fit are given in the table inserted. Lower panel: Residuals of the fit.

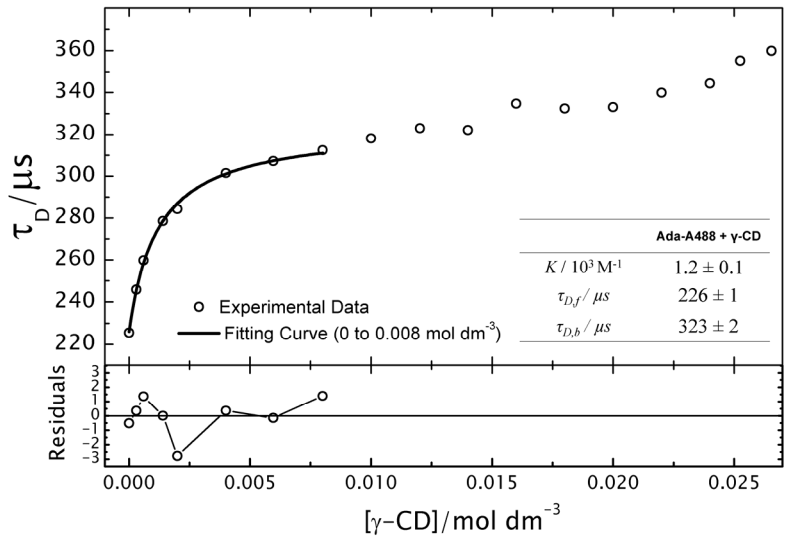

Figure 5. Upper panel: Mean diffusion times τ_D as function of γ-CD concentration determined from individual fits of the autocorrelation function to the normalized experimental correlation curves measured for γ-CD. The black line represents the fitted curve of 1:1 complexation model to τ_D up to 0.008 mol dm^{-3}. Parameters resulting from the fit are given in the table inserted. Lower panel: Residuals of the fit.

General experimental procedure

α-, β- and γ-CD (Sigma-Aldrich) were checked for fluorescence impurities and were found to be clean enough for classical fluorescence measurements and for FCS experiments. Water was purified with a Milli-Q system. The synthesis of the Ada-A488 compound is described elsewhere.[10]

Stock solutions of Ada-A488 were prepared as follows: the solid compound Ada-A488 was first dissolved in ethanol; then, an aliquot of this solution was diluted 1000 times adjusting the pH of the solution to 10. The concentrations of Ada-A488 in these stock solutions were still 25-fold higher than that necessary for the FCS measurements (approximately 10^{-9} mol dm^{-3}). The FCS samples were finally prepared by dilution of a constant volume of the corresponding Ada-A488 stock with different volumes of the freshly prepared CD stock solution (pH 10) and addition of water to adjust to a certain total volume. All these volumes were weighed so that concentration corrections could be performed. Special care was taken in order to avoid any possible contamination of the samples with fluorescent impurities. At the highest CD concentrations turbidity of the solutions was observed, specially for α- and γ-CD, that caused the appearance of an additional small very slow term in the correlation curves.

The confocal epi-illuminated setup used for the FCS measurements and the typical experimental conditions were described in previous articles.[6-10] The focal area and the detection volume were calibrated with Rhodamine 123 in aqueous solution at low irradiance yielding a radial $1/e^2$ radius of $w_{xy} = 0.53$ μm. The value of D(R123) = $(4.6 \pm 0.4)\times10^{-10}$ m^2s^{-1} is estimated from recent PFG-NMR [30] and dual-focus FCS [31] data. The diffusion coefficients are given for 25ºC. All given uncertainties correspond to one standard deviation from the fits and do not include calibration errors. Series of FCS curves measured at different host concentrations were analyzed by global "target" analysis programmed in OriginPro 8.0 (OriginLab Corporation, US). An empirical weighting function was used in order to take into account the strong variation of the noise in the FCS curves.

Acknowledgements

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