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Formation of Cyclodextrin Inclusion Complexes with Doxycyclin-Hyclate: NMR Investigation of Their Characterisation and Stability

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Abstract

The solubility of cyclodextrin (CD) can be notably improved when it is included in a polymeric structure. CD was reacted with citric acid, yielding a water-soluble polymer whose inclusion properties towards doxycyclin-hyclate (DOX) as guest molecule were investigated by NMR. The new DOSY (Diffusion Ordered SpectroscopY) method, based on diffusion coefficient measurements is convenient study complexes made of large molecules and it was applied to determine the association constants between DOX and parent β and γ-CD and their polymerised forms. The association constant obtained by DOSY was compared with that determined more classically by the chemical shift variation measurement using Scott's plot.

Introduction

Cyclodextrins (CDs) are polysaccharides made up of six to eight D-glucose units (α, β and γ-CD, respectively) connected at the C_1 and C_4 carbon atoms. They are able to form inclusion complexes with various guest molecules of suitable polarity and dimensions because of their special molecular structure: a hydrophobic internal cavity and a hydrophilic external surface [1–4].

This type of inclusion complex is generally able to reduce the volatility of fragrances and perfumes, to enhance the solubility of drugs, or to protect guests from thermal, chemical and oxidisation degradations. As a consequence, many applications have appeared over the last two decades in various industrial fields.

β-CD is the most widely used in the CD family, but it presents a solubility of only 1.85 g/100 mL at 25 °C in water, which is a limiting factor for its use. In addition, β-CD inclusion complexes also present low solubility and may result in a precipitate. Methyl or hydroxypropyl derivates of β CD which are more water soluble, can be used to replace their parent molecule. We recently proposed a new path for the synthesis of CD polymers (polyCD) which are very soluble products (in the range of 1 g mL^{-1}), even for those based on β-CD. It was necessary however to test if the polymerised CD cavities were still able to include a guest molecule, and to compare the stability of the complex with that of a native CD.

CDs, for example, are used to improve the solubility of active substances or to reduce the volatility of fragrances and perfumes. In particular, the use of CDs in the pharmaceutical fields aims at enhancing stability or at solubilising poorly soluble drugs to make them more bioavailable. Recently, we presented a new type of biomaterial with controlled drug delivery activity based on the release of antibiotics or biocides by grafted CDs.

NMR spectroscopy is the most widely used technique to study CD complexes [5–6]. This paper describes the NMR study of the inclusion of doxycyclin-hyclate (DOX) with parent and polymerised β-CD.

Firstly, the stoichiometry of the β-CD-DOX complex was investigated using the current Job plot method. Then the association constant was assessed using the phenomenon of chemical shift variation between free and complexed molecules following the method proposed by Scott.

Secondly, the polyCD-DOX system was investigated. In fact, as NMR resonance signals were broadened by the viscosity induced by the polymer in solution, it was not possible to follow the chemical shift variation of the DOX protons. We therefore applied another recent NMR method, called DOSY (Diffusion Ordered Spec-

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troscopY) based on diffusion coefficients, which are different for free and complexed molecules and whose measurements make it possible to determine the binding constant between β-CD, γ-CD, poly β-CD or poly γ-CD and DOX.

The values of the binding constants between DOX, native CD and polyCD, calculated with both methods are reported and discussed. At the same time, a dipolar interaction study was performed using the ROESY NMR sequence, to confirm the inclusion of DOX in the two systems.

Experimental part

Products

The synthesis of polyCD was previously reported [7–8] and is summarised as follows: CD, citric acid and the catalyst were solubilised in water. The solvent was then evaporated and the resulting mixture was treated at 140 °C under vacuum. Water was then added, and the solution was dialysed and lyophilised. The polyCD polymer was then collected in the form of a white powder.

Citric acid was purchased from Aldrich (Milwaukee, Wi, USA). β-CD (Kleptose®) was a gift from Roquette, Lestrem, France, and γ-CD (Cavamax W8) was a gift from Wacker Specialties (Burghausen, Germany).

NMR study

All NMR experiments were carried out on a Bruker AVANCE 300 spectrometer operating at 300.09 MHz and equipped with a multinuclear z-gradient inverse probehead and with a BGU-2 field gradient accessory capable of delivering z-field gradient up to 59 G cm^{-1}. In all experiments, the probe temperature was maintained at 298 K and standard 5 mm NMR tubes were used.

2D-ROESY spectra were recorded with a mixing time of 500 ms during the spin-lock.

All samples were prepared in phosphate buffer solution with deuterium oxide (pD = 7.24, 0.1 M)

Determination of stoichiometry

The stoichiometry of host-guest complexes was determined by the continuous variation method (Job's method) [9–12]. The total concentration of the interacting species in the solution was kept constant at 10 mM and 20 mM for β-CD and γ-CD respectively. The molar fraction r of the guest varied in the range 0.2–0.8.

Determination of apparent binding constants by chemical shift variation

The association constant (K_a) between CD and guest for a complex 1:1 is expressed by:

$$CD + G \rightleftarrows G{:}CD$$

$$K_a = \frac{[G:CD]}{[CD]\cdot[G]} \quad (1)$$

where [CD] is the concentration of free CD, [G] is the concentration of free guest, [G:CD] is the concentration of the inclusion complex.

K_a values were determined by applying Benesi–Hildebrandt's equation adapted to NMR by Scott [13–14], which, for a complex of 1:1 stoichiometry and in the case of a large excess of guest, is:

$$\frac{[CD]}{\Delta\delta_{ob}} = \frac{[CD]}{\Delta\delta_c} + \frac{1}{K_a\cdot\Delta\delta_c} \quad (2)$$

[CD] is the total molar concentration of CD, $\Delta\delta_{ob}$ is the chemical shift difference observed in a guest for a given [CD], $\Delta\delta_c$ is the difference in chemical shift between the complexed and the free guest at saturation. The slope of the plot for $[CD]/\Delta\delta_{ob}$ against [CD] for a fixed amount of substrate is thus equal to $1/\Delta\delta_c$ and the intercept with the vertical axis is equal to $1/K_a{\cdot}\Delta\delta_c$.

Determination of apparent binding constant by diffusion coefficients

DOSY (Diffusion Ordered SpectroscopY) experiments were performed using a stimulated echo sequence incorporating bipolar gradient pulses and a longitudinal eddy current delay (BPP-LED) [15]. The gradient strength was logarithmically incremented in 32 steps from 2% up to 95% of the maximum gradient strength. Diffusion times and gradient pulse durations were optimised for each experiment in order to achieve a 95% decrease in resonance intensity at the largest gradient amplitude; typically, diffusion times (Δ) between 60 and 100 ms, gradient strength (δ * 0.5) between 2.5 and 3.6 ms and spoil gradient strength of 6 ms. Gradient strength, g in the z direction was calibrated by $D_{H_2O} = 2.3\times10^{-9}$ m^2 s^{-1} at 298 K using a 90% H_2O/10% D_2O mixture. After Fourier transformation and baseline correction, the diffusion dimension of the 2D DOSY spectra was processed by means of the Bruker Xwinnmr software package (version 3.0).

The DOSY technique (Diffusion Ordered SpectroscopY) consists of a diffusion delay, flanked by two pulse-field gradients, where the magnetisation fraction rephased by the second pulse, is described by:

$$I = I_0\cdot\exp[-D(2\pi\gamma G\delta)^2(\Delta-\delta/3)] \quad (3)$$

where I is the signal strength at gradient strength G (T m^{-1}), D the diffusion coefficient (m^2 s^{-1}), γ the gyromagnetic ratio (rad T^{-1} s^{-1}), δ the gradient

duration (s) and Δ the time between the start of the two gradient pulses (s).

The diffusion constant depends on the size of the molecule:

$$D = \frac{KT}{6\pi\eta r} \tag{4}$$

where K is the Boltzmann constant (1.380662×10^{-23} J K^{-1}), T the absolute temperature (K), η the dynamic viscosity (Pa S) and r the radius of the molecule (m). In the case of non-spherical molecules, r is replaced by R_h the hydrodynamic radius [16–18].

The bound and free guests underwent fast exchange on the diffusion time scale and, therefore, the observed guest diffusion coefficient D_{obs} is the weighted average of the free solution D_{free} and CD bound $D_{complex}$ values, and is described in references [19–21]:

$$D_{obs} = \rho D_{complex} + (1 - \rho) D_{free} \tag{5}$$

where ρ the fraction of bound guest can be determined by:

$$\rho = \frac{D_{obs} - D_{free}}{D_{complex} - D_{free}} \tag{6}$$

The diffusion coefficients of the bound guest ($D_{complex}$) could not be measured and were taken to be equal to the diffusion of CD measured for the CD/guest solutions, which was assumed to be equal to that of the free CD ($D_{complex} \approx D_{CD}$).

The fraction of guest molecules ρ bound to CD is defined as:

$$\rho = \frac{[G:CD]}{[G] + [G:CD]} \tag{7}$$

where [G] is the concentration of free guest, [G:CD] is the concentration of the inclusion complex.

Combining Equations (1) and (7) gives

$$\rho = K_a \frac{[G] \cdot [CD]}{[G] + [G]K_a[\mathrm{CD}]} \tag{8}$$

$$\rho = K \frac{[CD]}{1 + K_a[CD]} \tag{9}$$

The diffusion coefficient of included molecule was measured with an increasing amount of CD which affected the viscosity of the solution and thereby the measured diffusion coefficient. This was evaluated by measuring HOD diffusion at each concentration and then correcting the measured value [22].

$$D_{cor} = D_{app} \cdot \frac{D_{HOD(ref)}}{D_{HOD(app)}} \tag{10}$$

where D_{app} stands for the measured diffusion constant, D_{cor} stands for viscosity corrected diffusion constant, $D_{HOD(ref)}$ the measured diffusion constant of the solvent in pure tampon solution and $D_{HOD(app)}$ the measured diffusion constant of the solvent with molecules.

Results and discussion

CD-DOX complex study

^{1}H NMR analysis and stoichiometry

Observation of the evolution in the chemical shifts of H-3 and 6′-Me protons of βCD and DOX respectively (Figure 1) induced by complexation, provided the first information about host-guest interactions.

The stoichiometry of the complexes was determined using the continuous variation method as described in the Experimental section. The Job plots relative to β-CD H-3 proton and to the methyl group substituent of the 6′ position of DOX (6′-Me) (Figure 2) showed symmetrical shaped plots with a maximum at $r = 0.5$ in both cases, indicating that the β-CD-DOX complex possessed 1:1 stoichiometry. The same result was obtained in the case of γ-CD.

Apparent binding constants

The binding constants were calculated from the chemical shift variation measured on complexation in a 0.5 mM solution of DOX in 0.1 mM phosphate buffer (pD = 7.24) and in the presence of increasing amounts of β-CD from 2 to 10 mM.

The γ-CD concentration was in the concentration range of 8–24 mM in the presence of 1 mM of DOX.

The ^{1}H NMR signal of the proton 6′-Me of DOX was used to calculate the binding constants.

From the $[CD]/\Delta\delta_{ob}$ *versus* [CD] plot, an apparent binding constant of 188 M^{-1} was calculated for β-CD (Figure 3) and 1820 M^{-1} for γ-CD.

These results can be interpreted in terms of cavity size : DOX fits better in γ-CD than in β-CD.

Diffusion coefficients

The apparent and corrected diffusion constants of CD and DOX were measured by the signals of protons H-3 and Me-6′ respectively in the presence of increasing amounts of CD. In such low concentration conditions,

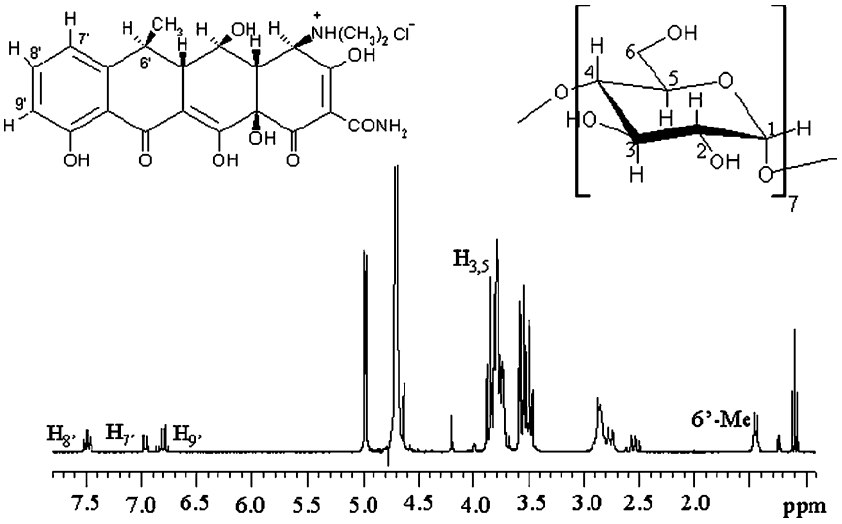

Figure 1. Structures of DOX, β-CD and spectrum of β-CD (5 mM) with DOX (5 mM).

viscosity correction is weak and the difference between apparent (D_{app}) and corrected diffusion (D_{corr}) constants is small, as shown in Table 1 for β-CD. The corrected diffusion constant of 2.28×10^{-10} m^2 s^{-1} is obtained for β-CD alone.

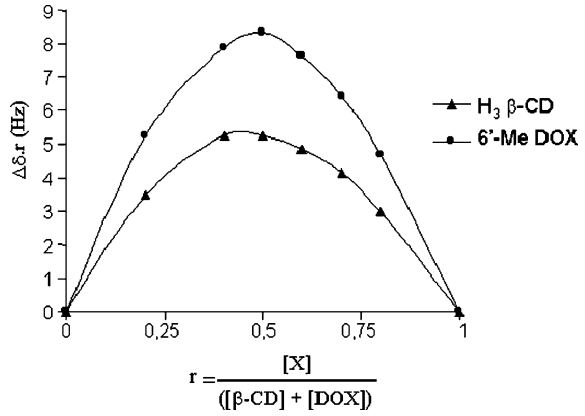

Figure 2. Job plots for β-CD/DOX complex, [x] = [DOX] and [βCD] for 6′Me and H-3 curves.

The fraction of bound DOX was calculated from this data according to Equation (6) (Table 1) and K_a can be determined by fitting the data to Equation (9) where ρ is plotted versus [CD] (Figure 4). K_a of 148 and 1540 M^{-1} were obtained for complexes with β-CD and γ-CD respectively.

These first results are close to those obtained by Scott's method mentioned above and confirm the validity of the diffusion method to determine the K_a value.

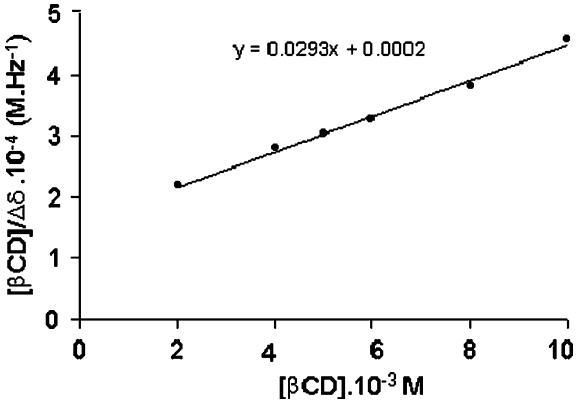

Figure 3. Scott plot of β-CD / DOX system.

PolyCD-DOX complex study

The NMR resonance signal was generally broadened in the presence of a polymer, and low resolution of the

Table 1. Apparent and corrected diffusion constants (10^{10} D m^2 s^{-1}) of DOX, β-CD and fractions of bound DOX (ρ) at different concentrations of β-CD, [DOX] = 0.5 mM, T = 298 K

[βCD] mM	D_{app} (βCD)	D_{app} (DOX)	D_{corr} (βCD)	D_{corr} (DOX)	ρ
0		3.78		3.57	
2	2.31	3.25	2.34	3.30	0.22
4	2.31	3.07	2.33	3.09	0.39
5	2.31	3.00	2.33	3.03	0.44
6	2.36	2.92	2.35	2.91	0.54
7	2.33	2.93	2.30	2.90	0.53
8	2.27	2.98	2.29	3.02	0.44
10	2.32	2.84	2.34	2.86	0.58

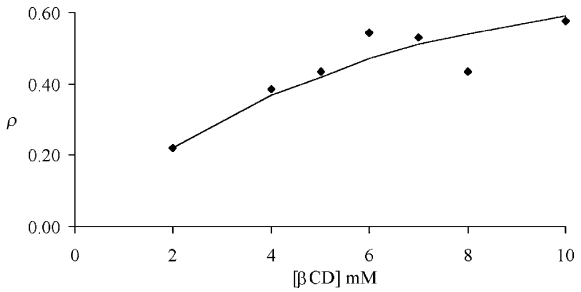

Figure 4. Plot of ρ versus the concentration of β-CD.

spectra prevented the determination of the association constant of DOX with polyCD according to the chemical shift variation method. Therefore, the DOSY approach was applied and the diffusion constants of polyCD and DOX determined as for native CD, as mentioned above. The diffusion constant values measured were corrected in respect of water diffusion constant according to the same equation as for the CD-DOX complex. The corrected diffusion constant of 0.95×10^{-10} m^2 s^{-1} was obtained for polyβ-CD alone. The results concerning the polyβ-CD/DOX system are presented in Table 2, the fraction of bound DOX was

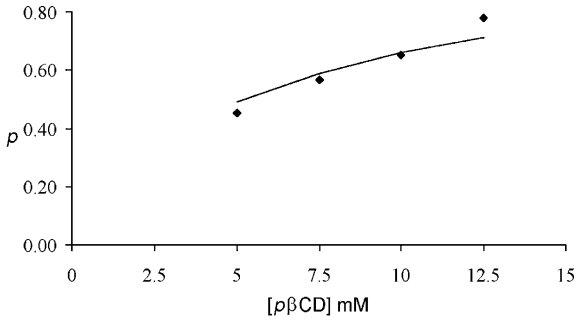

Figure 5. Plot of ρ versus the concentration of polyCD (expressed as a concentration of equivalent CD units).

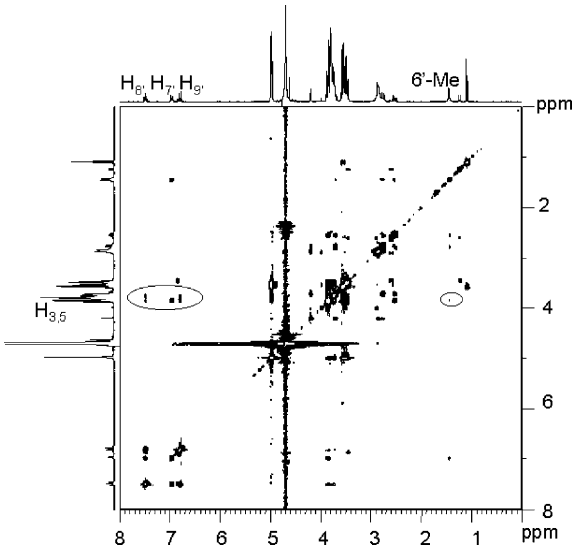

Figure 6. ROESY spectrum of β-CD (5 mM) with DOX (5 mM).

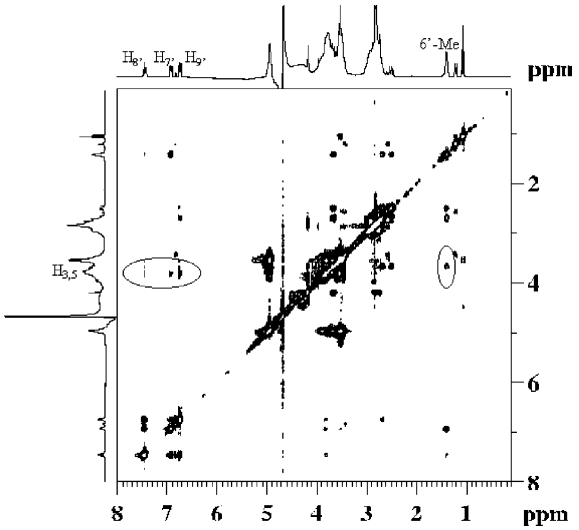

Figure 7. ROESY spectrum of polyCD (10 mM equivalent CD units) with DOX (10 mM).

calculated and K_a could be determined after drawing the curve-fitting from the data by plotting ρ versus the concentration of the equivalent CD concentration (Figure 5).

It is worth mentioning that a preliminary NMR investigation of polyCDs showed that CD moieties represented 50%-wt of the polymer (in polyβ-CD and polyγ-CD), so that the equivalent CD concentration of the polyCDs solutions could easily be determined.

K_a values of 192 and 1630 M^{-1} were obtained in both polyβ-CD and polyγ-CD systems through this DOSY method. The values are of the same order as those measured for the native CD/DOX systems mentioned above, as reported in Table 3.

Table 2. Apparent and corrected diffusion constants (10^{10} D m^2 s^{-1}) of DOX, β-CD and fraction of bound DOX (ρ) at different concentrations of polyCD (expressed as a concentration of equivalent CD units) [DOX] = 0.5 mM, T = 298 K

[CDunits] mM	D_{app} (polyβCD)	D_{app} (DOX)	D_{corr} (polyβCD)	D_{corr} (DOX)	ρ
0		3.78		3.57	
5	0.93	2.37	0.93	2.38	0.45
7.5	0.91	2.03	0.92	2.08	0.57
10	0.91	1.80	0.94	1.86	0.65
12.5	0.93	1.49	0.95	1.53	0.78

Table 3. Apparent binding constants K_a of DOX-CD complexes by two methods

	β-CD	Poly β-CD	γ-CD	Poly γ-CD
K_a (Scott) M^{-1}	188		1820	
K_a (DOSY) M^{-1}	148	192	1540	1630

2D ROESY

To confirm DOX inclusion in the CD cavity, ROESY experiments were performed with native and polymerised cyclodextrins. In ROESY for native and polymerised β-CD (Figures 6 and 7), we observed cross-peaks connecting the H-7′,8′,9′ and also 6′-Me resonance of DOX to the H-3 and 5 that are characteristic of the interior of the β-CD cavity. This also indicates that DOX was included in the CD cavity by its aromatic side. The same observations were made in the cases of native and polymerised γ-CD with the DOX system.

Furthermore, the results obtained by the two methods (DOSY and Scott), for native CDs and their polymerised forms, also concord. This confirms the validity of the DOSY technique, and that polymerisation of CDs by crosslinking with citric acid does not affect the apparent binding constants with this substrate. The polymerisation of cyclodextrins did not enhance the binding constants. This proves that cooperative binding of DOX with CD polymer cavities was not observed [23].

Conclusion

The objective of this paper was to investigate by different NMR techniques the complexation of DOX with native and polymerised CDs. The results showed that the innovative DOSY technique is available, complementary and also practical, especially in the case of a study of polymeric systems. The results also highlighted that polymerised cavities were still accessible to the substrate despite the structure of the crosslinked polymer network.

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