Inclusion complexes of cyclodextrins with nitroxide-based spin probes in aqueous solutions

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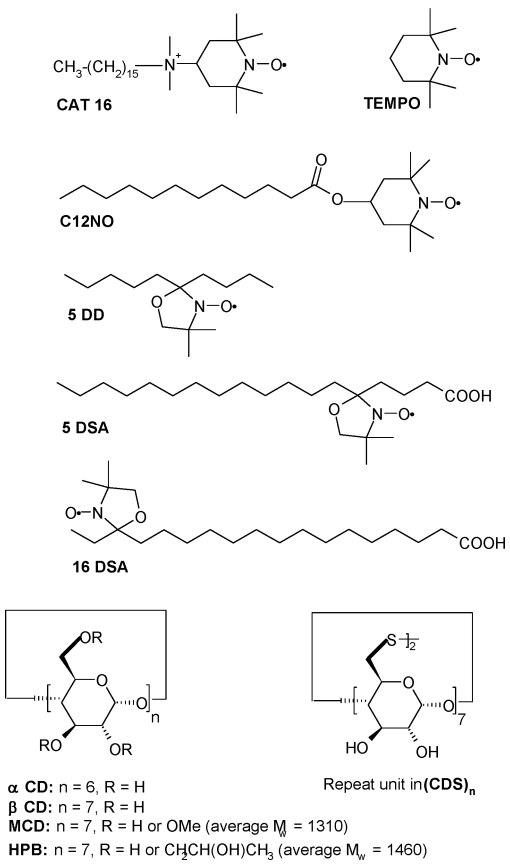
Formation of inclusion complexes between several cyclodextrin derivatives and TEMPO and DOXYL-based spin probes was studied by EPR spectroscopy. Competition between alkyl chains and nitroxide functionalities for cyclodextrin cavities leads to different types of complexation. Long alkyl chains in amphiphilic spin probes interact preferentially with cyclodextrins, and TEMPO units in such molecules are unaffected by complexation. DOXYL-type spin probes however form stronger complexes with cyclodextrins; this complexation changes hyperfine splitting and tumbling rate of the nitroxide group. Comparison of EPR spectra of free cyclodextrin and cyclodextrin-based polymeric nanocapsules made it possible to assess the tumbling of the spin probe inside the cyclodextrin units without the contribution of the tumbling of the whole complex.

Introduction

Cyclodextrins (CDs) are cyclic molecules containing at least 6 glucopyranose units, which adopt a truncated cone conformation with two hydrophilic rims decorated by primary and secondary hydroxyl groups and a relatively hydrophobic cavity. These features result in their ability to form inclusion complexes with hydrophobic molecules in aqueous solutions or in the solid state.[1] The presence of hydroxyl groups in the CD structure gives the opportunity to functionalise naturally occurring CDs (*e.g.* α-CD and β-CD) and hence to tune their properties and use them as building blocks for making supramolecular systems. By functionalising CDs, it is possible to obtain compounds with enhanced affinity for specific species; such selective hosts can find applications in drug delivery processes. For example, CDs functionalised with amphiphilic groups at the primary and secondary rims often self-organise into micelles, nanoparticles or vesicles, and these aggregates may act as transport vectors for hydrophobic and hydrophilic guests.[2–4] We have recently reported the preparation of polymeric CD-based nanocapsules formed by crosslinking of perthio-β-CD in water in the presence of air. The aggregates retained their ability to encapsulate and release guest molecules.[5]

Inclusion complexes of CDs have been investigated by various physico-chemical methods. For instance, EPR spectroscopy has been successfully used to characterise inclusion complexes of persistent or short lived radicals with CDs, providing some structural and thermodynamic information about inclusion complexes.[6–14] Spin probes can also be chemically attached to the CD units;[15–20] however applications of these materials are limited by complicated synthesis and self-inclusion of the paramagnetic moiety in the CD cavity.

In this paper, we analyse changes in EPR parameters of a range of spin probes (Scheme 1) observed during complexation with CDs. The use of different spin probes enabled us to establish different types of complexation of hydrophobic guests with CDs, depending on the polarity and geometrical features of the interacting species. We used commercially available cyclodextrins α-CD, β-CD, hydroxypropyl-β-CD (HPB), methyl β-CD (MCD)



Scheme 1 Structures of cyclodextrins and spin probes used in this work.

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and water-soluble, disulfide-crosslinked, polymeric β-CD-based nanocapsules [$(CDS)_n$]. The latter materials (average diameter *ca.* 30 nm) are spontaneously formed during air oxidation of an aqueous suspension of per-thiolated β-CD.[5]

Results and discussion

Inclusion of nitroxide-based spin probes into CD cavities mainly affects two EPR parameters of the probes: the nitrogen hyperfine splitting (a_N) which reports on the polarity around the probe (*e.g.*, water *vs.* CD cavity) and line width which depends on the rate of tumbling (τ). The tumbling of the spin probe is often slowed down upon complexation. However, EPR spectroscopy is not always sensitive to the formation of inclusion complexes. If the nitroxide group in the complex is surrounded by water molecules, changes in local polarity could be very small. Tumbling includes contribution from diffusion of the whole complex and diffusion of the spin probe within the complex. If the overall tumbling rate of the complexed probe remains high, the changes to the EPR spectrum upon complexation may be small. We found however that accurate simulation of spectral shape makes it possible to determine the formation of inclusion complexes in most cases.

Stability of CD inclusion complexes is determined in the first instance by the geometric properties of the CD cavity (which are related to the number of glycosidic units) and by the size of guest molecules.[1] Additionally, the inclusion process includes desolvation of the cavity and the guest molecule,[21] and therefore hydrophobic/hydrophilic groups in the structure of the host and the guest can also significantly affect the complexation.

Binding constants for complexes of CDs with nitroxide type radicals are usually in the range 10^2–10^4 M^{-1}.[22,23] At the concentrations of reagents used in this study ([CD] = 10^{-2}–10^{-3} M; [probe] = 10^{-4} M), complete binding is expected for guests with high affinity for CDs. Weaker binding guests will result in a mixture of bound and unbound species. The deconvolution of the EPR spectra into individual components in these cases makes it possible to estimate the strength of binding.

Interaction of spin probes bearing TEMPO moiety with cyclodextrins

EPR spectra of TEMPO radicals suggest that a host–guest interaction with α-CD does not occur, as the spectra are indistinguishable from those of a free probe even at high concentration of α-CD (10^{-2} M). In the presence of β-CD, the changes to the EPR spectrum are apparent (Fig. 1). In particular, the asymmetry of the high field line suggests the co-existence of two environments around the paramagnetic group.

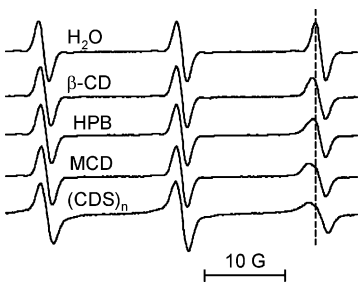

Fig. 1 EPR spectra of TEMPO in water, in β-CD, HPB, MCD and $(CDS)_n$ solutions. Concentration of all cyclodextrins was 1 mM.

Table 1 EPR parameters for complexed TEMPO in 1 mM solutions of functionalised cyclodextrins

Cyclodextrin	a_N (G)	$\tau \times 10^{10}$ (s)	Free/complexed ratio
β-CD	16.77	0.84	1.24
HPB	16.70	1.60	0.94
MCD	16.56	1.36	0.84
$(CDS)_n$	16.77	7.82	0.15

In the presence of functionalised cyclodextrins, the asymmetry of the high field line became more evident. Spectral parameters for individual components were obtained by simulation. In all cases, we found that the spectra can be fitted as a mixture of a spectrum of TEMPO in pure water (a_N = 17.33 G, $\tau = 0.63 \times 10^{-10}$ s) and another component with a smaller hyperfine value and a slower tumbling rate. The spectral parameters and ratios of the two components are shown in Table 1.

The EPR parameters of the slow component (Table 1) are consistent with the cyclodextrin-included TEMPO. The nitroxide group in these complexes is probably located inside the CD cavity. This is in agreement with the large changes in the hyperfine constant upon complexation (caused by significant reduction of polarity) and the drop in the tumbling rate. The EPR parameters also depend on the size and hydrophobicity of different cyclodextrins. For instance, addition of hydroxypropyl and methyl groups to β-CD leads to progressive reduction of polarity which is seen as a drop in a_N values (Table 1). Similarly, CD functionalisation increases the size of the host which leads to the reduction of tumbling rates of the overall complex.

From the component ratios (Table 1), binding constants of TEMPO with derivatised CDs were estimated as 8×10^2, 1.1×10^3, and 1.2×10^3 M for β-CD, HPB, MCD, respectively. The binding constant for β-CD is somewhat smaller than the literature value obtained by a similar EPR approach at Q-band.[23] The accuracy of the simulation is decreased if the complexation kinetics are comparable with the EPR frequency; the discrepancy is likely to result from such errors.

In order to distinguish the tumbling of the whole complex from the tumbling of the spin probe inside the CD unit, we have investigated the complexation of spin probes with polymeric nanocapsules composed of β-CD units cross-linked *via* disulfide bonds $(CDS)_n$. While the motion of the spin probe inside the CD cavity in these polymers should be comparable to that of monomeric β-CD, the tumbling of the overall nanocapsule is negligible on the EPR timescale. The local motion of the CD units in the cross-linked polymer is also expected to be very slow. The significantly reduced tumbling rate of the slow component for the TEMPO complex with the polymer (Table 1) suggests rather limited mobility of the guest inside the host, consistent with encapsulation of the nitroxide group within the CD cavity. The binding constant of TEMPO with the polymer was estimated as 7×10^3 M^{-1}; the stronger complexation is probably due to the more hydrophobic environment of the thiolated CD cavity.

CAT16 and C12NO spin probes show a different type of interaction with CDs. Fig. 2 shows EPR spectra of CAT16 in water and aqueous 0.01 M β-CD. Only a small decrease of a_N value (0.13 G) in the presence of β-CD was observed suggesting that

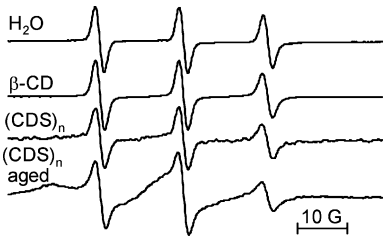

Fig. 2 EPR spectra of CAT16 in water, β-CD (10^{-2} M) and fresh and aged $(CDS)_n$ solutions.

CD forms a complex with the hydrophobic alkyl chain rather than the nitroxide group. This is also supported by the relatively small changes in the τ value upon complexation (Table 2). The lack of inclusion of the TEMPO group into CD units could be explained by the hydrophilic cationic environment near the nitroxide group in CAT16 probe, and the preferential complexation of CDs with the aliphatic chains which are the most hydrophobic groups in this molecule.

A similar effect was observed with the polymeric nanocapsules $(CDS)_n$ (Fig. 2). Virtually no change in the a_N upon complexation with CAT16 can be detected, while the increase in the τ value was quite modest (Table 2). Presumably, all CDs bind the most hydrophobic part of this amphiphilic spin probe, *e.g.*, by threading the aliphatic chain through the CD cavity. Interestingly, ageing of the polymer suspension significantly changes the appearance of the spectrum, with a very slowly tumbling component clearly visible (Fig. 2). The appearance of immobilised species during ageing was also observed with other spin probes. This is most likely due to the aggregation of the nanocapsules. Precipitation after several days of ageing was previously observed and was tentatively explained by the redistribution of the molecules in the polymer *via* disulfide exchange.[5] Presumably, EPR is quite sensitive to this process as formation of even small aggregates significantly slows down the tumbling and enables binding of guest molecules between the CD units.

Table 2 EPR parameters for CAT16 with functionalised cyclodextrins

Sample	a_N (G)	$\tau \times 10^{-10}$ (s)
H_2O	16.83	1.42
β-CD[a]	16.70	2.72
$(CDS)_n$[b]	16.74	4.60

[a] [CD] = 10 mM. [b] [CD] = 1 mM.

Table 3 EPR parameters for C12NO in 1 mM solutions of functionalised cyclodextrins

Cyclodextrin	a_N (G)	$\tau \times 10^{-10}$ (s)
α-CD	16.93	2.69
β-CD	16.88	1.77
$(CDS)_n$	16.81	3.49
HPB	16.90	2.76
MCD	16.76	2.47

C12NO is not soluble in water in the absence of CDs. In the presence of CDs, the EPR signal for C12NO increases which suggests formation of inclusion complexes (Table 3). One can see that the changes in the hyperfine constant caused by this complexation are relatively small, suggesting that the binding again only affects the hydrophobic tail of the molecule. This is confirmed by the high mobility of the complexed spin probe. Even inclusion in the polymeric CD nanocapsules does not significantly increase the τ value. Complexation of the hydrophobic tail is also consistent with the observed binding to α-CD, as this cyclodextrin does not interact with the TEMPO unit.

Interaction of cyclodextrins with DOXYL spin probes

Unlike CAT16 and C12NO amphiphilic probes, doxylstearic acids 5-DSA and 16-DSA have the nitroxide unit in the middle of the hydrophobic chain. Therefore, interaction of CDs with these probes (presumably involving the hydrophobic chain) is expected to affect the environment around the spin probe. This hypothesis is confirmed by the data in Table 4. Complexation with CDs significantly perturbs the hyperfine constants of both spin probes. Significant increases in τ value are also consistent with the immobilisation of the nitroxide during complexation.

An EPR study of the complexation of β-CD with 5-DSA and 16-DSA has been recently reported.[24] The authors observed two-component spectra at higher CD concentration, with neither component corresponding to the free probe in water. These results were interpreted in terms of formation of a mixture of 1:1 and 1:2 complexes of spin probe:CD. In both complexes, the complexation

Table 4 EPR parameters of 5- and 16-DOXYL stearic acids and 5-DOXYL decane in water and functionalised cyclodextrin solutions

		5-DSA		16-DSA		5-DD	
Sample	[CD], M	a_N (G)	$\tau \times 10^{10}$ (s)	a_N (G)	$\tau \times 10^{10}$ (s)	a_N (G)	$\tau \times 10^{10}$ (s)
H_2O	0	15.70	2.61	15.80	1.31	15.86	1.50
α-CD	10^{-3}	15.55	7.27	15.60	4.58	15.76	3.20
β-CD	10^{-3}	15.57	4.79	15.62	5.23	Free + complex[a]	
β-CD	10^{-2}	Two complexes[a]		Two complexes[a]		15.26	5.71
HPB	10^{-3}	15.60	6.55	15.55	4.33	Free + complex[a]	
HPB	10^{-2}	15.55	7.71	15.52	5.59	15.32	7.80
MCD	10^{-3}	15.52	6.66	Two complexes[a]		15.37	6.42
MCD	10^{-2}	15.42	8.97	15.50	9.10	15.14	6.73
$(CDS)_n$	10^{-3}	15.21	15.99	15.3	9	15.17	16.87

[a] Two component spectra which include contributions from free and/or complexed spin probes as indicated.

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was suggested to occur *via* threading of the hydrophobic group through the CD cavity. Our results are in agreement with this study, and spectral data for β-CD complexes with 5-DSA and 16-DSA are similar to those reported. We note that unlike TEMPO derivatives, DOXYL compounds form inclusion complexes with α-CD. This further supports the proposed the threading of the hydrophobic chain through the CD cavity in the complexes of the DOXYL derivatives.

The trends in the EPR parameters of the spectra recorded in the presence of different CDs are similar to those observed with the TEMPO-based spin probes. Increased τ value in the order β-CD < HPB < MCD is consistent with the increased size of the cyclodextrins which results in the reduced tumbling. The hyperfine constants also parallel the polarity of the host CD.

The interaction of DOXYL type spin probes with CDs is quite strong, as even at the concentration of CDs at 10^{-3} M there is no evidence of the free spin probe in solution. The presence of two components in some spectra (Fig. 3) is thus only consistent with the formation of higher stoichiometry complexes. This is also in agreement with the progressive immobilisation of the spin probe at the higher CD concentration. This effect seems to be particularly strong for MCD, as only the slowest component was observed at 0.01 M concentration (Tables 4, 5).

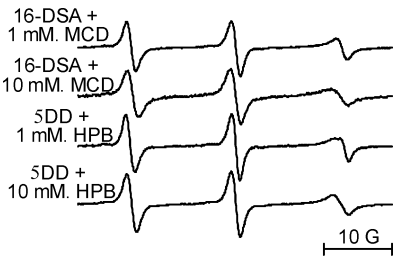

Fig. 3 EPR spectra of doxyl-based spin probes in cyclodextrin solutions.

The 5DD spin probe showed rather different behaviour. Unlike 5- and 16DSA, the two component spectra for complexation of 5DD with β-CD and HPB (observed only at 1 mM cyclodextrin concentration) can be assigned to a mixture of free 5DD and the inclusion complex (Tables 4, 5). We conclude therefore that the complexation of the 5DD probe with CDs is weaker than that of DOXYL-stearic acids (as not all 5DD is complexed at 1 mM cyclodextrin concentration). This is consistent with the shorter hydrophobic chain of the 5DD probe, presumably weakening the interactions with the CDs. We also note significantly higher τ values for 5DD complexes as compared to other DOXYL derivatives. Such increased immobilisation could be tentatively explained by the encapsulation of the DOXYL group inside the CD cavity (*e.g.*, as opposed to the threading the hydrophobic chain through the CD cavity) in the complex between 5DD with CDs. This complexation mode could be driven by the weakened interaction of the short alkane chains with the CD units. Rather big changes in the hyperfine constant observed upon complexation of 5DD with CDs, are also in agreement with the possible incorporation of the DOXYL group in the CD cavity in this case. Binding constants for 5DD with β-CD and HPB were estimated as 1.7×10^3 and 4.3×10^3 M^{-1}, respectively.

Table 5 EPR parameters for individual components for DOXYL-based spin probes in 1 mM solution of functionalised cyclodextrins

	Fast component		Slow component		
Sample	a_N (G)	$\tau \times 10^{10}$ (s)	a_N (G)	$\tau \times 10^{10}$ (s)	Fast/slow
5DD-β-CD	15.86	1.50	15.30	4.65	0.58
5DD-HPB	15.86	1.50	15.20	7.00	0.23
16DSA-MCD	15.50	3.20	15.00	9.13	0.47

Formation of inclusion complexes of polymeric CD with all DOXYL-based spin probes leads to remarkably small hyperfine constants. This significant reduction in the a_N value as compared to the complexes of unmodified β-CD can only be explained by the presence of disulfide bridges on the lower rim of CD units in the polymer. Presumably in these complexes the nitroxide group is located in the hydrophobic microenvironment. The τ values in all cases are also significantly higher than for unmodified β-CD.

Conclusion

We have explored formation of inclusion complexes between a range of spin probes with several different cyclodextrins using EPR spectroscopy. Some clear trends emerged from the comparison of the EPR data. The properties of functionalised CDs determine the EPR parameters of the spin probes in the complexes. CDs with hydrophobic groups such as methyl or hydroxypropyl create a more hydrophobic microenvironment around the spin probes than the unmodified cyclodextrin. This results in the reduction of hyperfine constant. Functionalisation of CDs with large groups slow down the molecular tumbling of the inclusion complexes with the spin probes which leads to the increased τ values.

The TEMPO unit forms relatively weak complexes with all cyclodextrins. The TEMPO unit at the hydrophilic head group in amphiphilic spin probes does not form inclusion complexes with CDs at all; instead, complexation occurs *via* threading of the alkane chain through the CD cavity. This type of complexation only slightly affects the EPR spectra.

The DOXYL-based amphiphilic spin probes form strong complexes with all CDs. These complexes are likely to be formed by threading of the aliphatic chain through the CD cavity, consistent with the recent literature suggestion.[24] However, as the spin probe in this case is located in the middle of the hydrophobic tail, the complexation significantly affects the EPR parameters of the probe. On the other hand, in 5-DOXYL decane, the hydrophobic tails are too short to form strong complexes with the CD units. As a result, the complexation is significantly weaker, and likely occurs *via* inclusion of the nitroxide group in the CD cavity.

The comparison of the EPR spectra of unfunctionalised β-cyclodextrin with the crosslinked polymeric nanocapsules prepared from perthiolated β-cyclodextrin, made it possible to analyse local motion of the spin probes free from the contribution of the tumbling of the whole complex. The tumbling of the spin probes in polymeric cyclodextrin was significantly slower than the tumbling of the inclusion complexes with unfunctionalised cyclodextrin. In the case of amphiphilic spin probes with the TEMPO in the head group, the complexation leads to a modest reduction in probe mobility, as the CD unit threading the hydrophobic tail is relatively far from the nitroxide group. On the other hand, inclusion of the nitroxide group into the CD cavity, or complexation with the

hydrophobic chain attached to the nitroxide, leads to significant loss of mobility.

Experimental

Spin probes 5- and 16-DOXYL-stearic acids (5-DSA and 16-DSA), 5-DOXYL-decane (5DD), TEMPO were purchased from Sigma, and CAT16 was obtained from Molecular Probes, α-CD, β-CD, hydroxypropyl-β-CD (HPB, degree of functionalisation *ca.* 80%, average $M_w = 1460$) and methyl-β-CD (MCD, degree of methylation *ca.* 54–67%, average $M_w = 1310$) were purchased from Aldrich. The polymeric β-CD nanocapsules were synthesised as reported elsewhere. Briefly, perthiolated βCD was prepared following a literature procedure.[25] Perthiolated β-CD was then stirred in water for 15 h; insoluble material was removed by filtration, and the solvent was evaporated under reduced pressure to obtain the soluble nanoparticles.[5]

C12NO was prepared by reaction between hydroxy-TEMPO and dodecanoyl chloride in presence of pyridine by a modified literature recipe.[26] Briefly, 4-hydroxy-TEMPO (1 mmol, 172 mg) was mixed with dodecanoyl chloride (1.2 mmol, 261 mg) in 50 ml DCM in the presence of pyridine (1 ml). The mixture was stirred at room temperature for one day. Then, the reaction mixture was washed successively with 1 M HCl, saturated $NaHCO_3$, and water. The organic layer was dried over anhydrous Na_2SO_4. Solution was concentrated and purified on silica gel preparative TLC plates using 95:5 (v/v) DCM/ethyl acetate ($R_f = 0.8$). Yield 60%.

Stock solution for each spin probe was prepared in ethanol at 10^{-2} M concentration. To prepare samples for EPR measurements, an appropriate volume of ethanol solution was evaporated. The residue was then dissolved in distilled water or a CD solution in distilled water with appropriate concentration (10^{-3} M or 10^{-2} M) to make a solution containing *ca.* 10^{-4} M spin probe. The solutions were then transferred to glass capillaries and sealed prior to recording EPR spectra.

The EPR spectra were recorded at room temperature on a JEOL FA 100 spectrometer with 100 kHz modulation frequency, 0.998 mW microwave power, 480 s sweep time, 0.7 G modulation amplitude, time constant 0.3 s. Simulations of EPR spectra in the fast motion regime were carried out using EWVOIGTN program developed by Prof. A. I. Smirnov.[27] This program enables deconvolution of nitroxide spectra containing two components provided their hyperfine values are sufficiently different. The program simulates pseudo-Voigt spectral shape for nitroxides in fast motion and includes powerful optimisation algorithm. The built-in phase and baseline correction and ^{13}C satellite contribution provide more accurate deconvolution than other programs.

Binding constants were calculated by fitting the ratios of free and complexed spin probe to eqn (1).

$$\frac{TCD}{T} = \frac{2KT_0}{KCD_0 + KT_0 + 1 + \sqrt{(KCD_0 + KT_0 + 1)^2 - 4K^2T_0CD_0}} - 1 \quad (1)$$

Here TCD/T is the ratio of complexed and free nitroxide, respectively, K is the equilibrium constant, CD_0 and T_0 are initial concentrations of cyclodextrin and nitroxide, respectively.

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