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Mobility of spin probes in viscous cyclodextrin solutions†

Gabriela Ionita[a] and Victor Chechik*[b]

Received 17th November 2009, Accepted 30th March 2010
First published as an Advance Article on the web 11th May 2010
DOI: 10.1039/b924092f

Published on 11 May 2010. Downloaded by Michigan State University on 27/01/2016 02:07:15.

TEMPO-based spin probes and β-cyclodextrin form 1 : 2 guest–host complexes in viscous aqueous glycerol solutions at low temperatures. The spin probe mobility in these complexes is much higher than in the bulk glassy solution. The similar tumbling rates of the spin probe in viscous host–guest complexes and in solvent-free complexes suggest that the complexation effectively shields the nitroxide unit from the solvent molecules. This is consistent with the low activation barrier for molecular tumbling of immobilised label.

Introduction

EPR spectroscopy is often used to study cyclodextrin (CD) complexes with persistent free radicals in order to obtain structural and thermodynamic information about such systems.[1–9] In many cases, the magnetic parameters of free radicals change upon formation of a host–guest complex with CDs thus making it possible to directly study the complexation process. For instance, incorporation of a nitroxide radical into the CD cavity usually results in the reduction of nitrogen hyperfine splitting (a_N) due to a less polar environment around the probe. The complexed free radicals also often show slower rotational motion. We have recently used EPR spectroscopy to investigate complexation of a series of spin probes with CDs.[10] We found that, at room temperature, several spin probes bearing TEMPO unit did not form strong complex with CDs. For instance, complexation of TEMPO functionalised with a long alkane chain occurred by threading the alkane chain through the host cavity, rather than by encapsulation of the nitroxide unit.

In some cases, the relatively weak binding of nitroxides to CDs is further complicated by rather small differences in EPR parameters of complexed and uncomplexed nitroxide. This could make EPR insensitive to the host–guest complex formation. For instance, we have recently showed that ESEEM spectroscopy (which reports on the magnetic interactions between the nitroxide and magnetic nuclei, *e.g.*, deuterium in deuterated solvent) is more sensitive to the complexation than conventional EPR.[11] ESEEM experiments were carried out at low temperature with frozen solutions, and we noticed that the binding constant for host–guest complexes increased significantly at lower temperature. This prompted us to investigate the temperature dependence of host–guest interactions between nitroxide spin probes and CDs using conventional EPR. We reasoned that the reduced tumbling rates of the complex and free nitroxide at lower temperatures might help deconvolute the spectra into individual components. As pure water crystallises at 0 °C, we added 20% (w/w) glycerol in order to expand the temperature range. We have chosen several TEMPO derivatives with similar size but different substituents as spin probes (Fig. 1). All experiments were carried out by continuous wave EPR spectrometry operating at X-band.

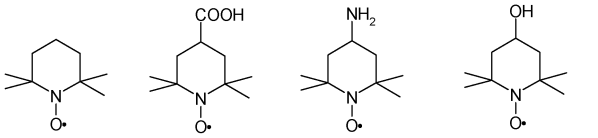

Fig. 1 Structures of spin probes used in this work.

Results and discussion

Inclusion complexes in aqueous glycerol below room temperature

The EPR spectra of spin probes (Fig. 1) in aqueous glycerol at temperatures below 270 K showed progressive reduction in their tumbling rate. The freezing of motion on the EPR time scale (powder pattern) was reached at slightly different temperatures for different probes (in the range 230–210 K), suggesting that rotational diffusion depends not only on the local viscosity but also on the specific interactions between the spin probes and the environment. For instance, rotational diffusion of TEMPO (**T**) slows down below EPR detection limit at a lower temperature than that for the other spin probes, probably due to the absence of functional groups capable of forming hydrogen bonds with the solvent molecules. At 210 K, however, all spin probes used in this study showed powder pattern EPR spectra. These observations are consistent with the literature[12] data on empirical cross-over temperature (T_{50G})[13a] for water : glycerol (8 : 2 w/w) mixture. Although this temperature is somewhat above glass transition temperature, the rotational diffusion of spin probes is already strongly inhibited.[12]

[a] *Laboratory of Quantum Chemistry and Molecular Structure, Institute of Physical Chemistry "Ilie Murgulescu", Splaiul Independentei 202, Bucharest, 060021, Romania. E-mail: gabi2ionita@yahoo.com*
[b] *Department of Chemistry, University of York, Heslington, York, YO10 5DD, UK. E-mail: vc4@york.ac.uk*

† Electronic supplementary information (ESI) available: Additional spectral data and simulation details. See DOI: 10.1039/b924092f

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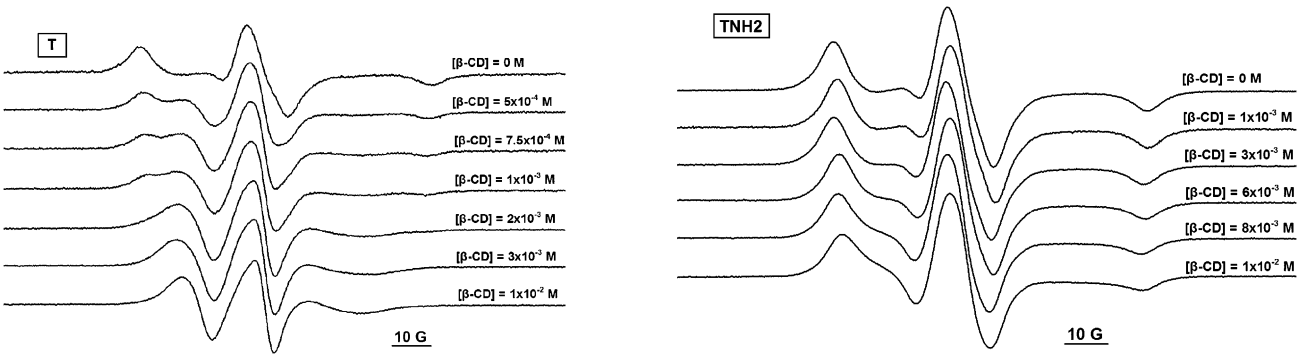

Fig. 2 EPR spectra of spin probes in water : glycerol (8 : 2 w/w) at different β-CD concentrations at 210 K.

Addition of β-CD to the solutions of spin probes at room temperature only slightly affected their EPR spectra (*e.g.*, a slight decrease in a_N value and reduction of the high field line intensity with increased concentration of β-CD were observed). Below room temperature, however, EPR spectra of spin probes became more sensitive to complexation. At temperatures below *ca.* 230 K the presence of two components (assigned to uncomplexed and complexed spin probes) is clearly visible in the spectra. Interestingly, the spectra at low temperature contain a component which tumbles much faster than pure **T** under the same conditions. For instance, free **T** shows a powder EPR spectrum at 210 K, but the spectrum in the presence of 10^{-2} M β-CD is dominated by a much more mobile component (Fig. 2). This suggests that the tumbling rate of the CD-included **T** is faster than that of free **T**. This is a rather unusual observation, as formation of host–guest complexes normally results in the reduction of the tumbling rate. Our results thus suggest that as the solution outside the CD molecules becomes increasingly viscous at low temperature, the environment inside the CD cavities remains quite mobile. This situation could be compared with gels: in a gel, solid immobile fibres hold the whole structure together while the voids between the fibres contain mobile solution. In our system, the CD-containing solvent matrix is nearly immobile while the guest molecules inside the CD cavities remain mobile.

We next recorded EPR spectra of different spin probes in solutions containing different concentration of β-CD at 210 K (Fig. 2 and ESI†). For **T**, **TOH** and **CT**, the mobile component dominates the spectra at high CD concentration. The ratio of mobile to immobile components changes with CD concentration, consistent with our assignment. As host–guest complexation is a temperature-dependent equilibrium, we tested whether the equilibrium was reached by recording EPR spectra at different time intervals. In all cases, the ratio of two components in the EPR spectra changed very rapidly (*e.g.*, within 10 min) after temperature adjustment but no further changes were observed after a 1 h interval.† This suggested that the equilibrium was reached quickly. It is interesting to note that the spectra of **TNH_2** probe do not show the mobile component at all; the immobile component however changes with the increased CD concentration. This suggests that while **TNH_2** forms inclusion complexes just like other probes, the mobility of included **TNH_2** is significantly lower than that of the other spin probes.

As both **CT** and **TNH_2** possess ionisable groups, proton transfer equilibrium could affect the mobility of the included complexes. Spectra in Fig. 2 were recorded without pH control; therefore, we also recorded EPR spectra of both spin probes in pH 2 and 10 buffers at 210 K. Surprisingly, the spectra were very similar to those recorded without pH control.† Definite conclusion about the effect of proton transfer equilibrium on the mobility of the encapsulated guests, however, cannot be made, as the pK_a of CD-included **CT** and **TNH_2** at 210 K is not known.

The ratios of free and complexed components in the spectra in Fig. 2 were obtained by fitting the experimental spectra to the mixture of two components: a free and an encapsulated probe. Fitting was carried out using a least squares method. These ratios could be used to estimate the binding constants of the guest molecules to β-CD at 210 K. Interestingly, experimental data were not consistent with 1 : 1 spin probe to CD stoichiometry. Good agreement with a theoretical equation, however, was obtained assuming 1 : 2 stoichiometry.† 1 : 2 complexes are fairly common for β-CD.[13b] The equilibrium constants thus calculated were 2.3×10^7, 1.2×10^5, 4.2×10^5 and 3.2×10^4 M^{-2} for **T**, **CT**, **TOH** and **TNH_2**, respectively.

The EPR spectra of frozen solution of **T** in 10^{-2} M β-CD are thus dominated by the **T** : β-CD complex, and the contribution of uncomplexed **T** can be ignored for this mixture. Fig. 3 shows the temperature dependence of the EPR spectra of this complex in the 130–230 K temperature range in water : glycerol solution. The change in the dynamics

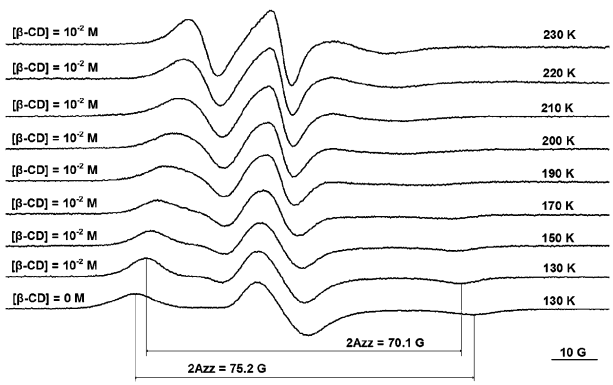

Fig. 3 EPR spectra of **T** in 8 : 2 (w/w) water : glycerol solutions of β-CD (10^{-2} M) at different temperatures and in the absence of β-CD in water/glycerol at 130 K.

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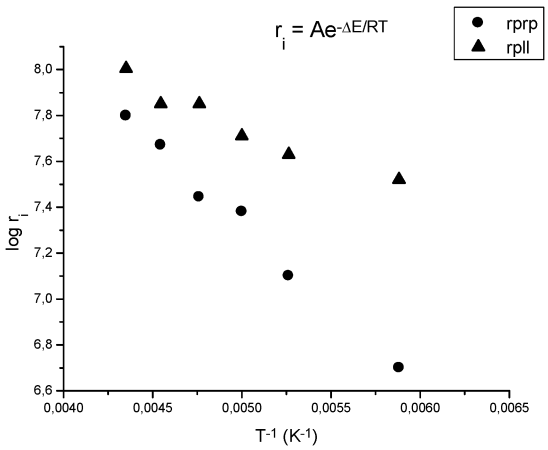

Fig. 4 Arrhenius plot for the rotational diffusion rates of **T** in β-CD complex.

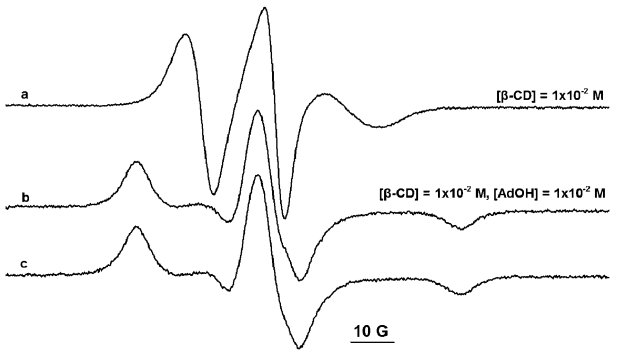

Fig. 5 Competition experiment: EPR spectra of **T** at 210 K in 8 : 2 (w/w) water : glycerol mixture in the presence of β-CD (10^{-2} M) (a), β-CD (10^{-2} M) + adamantol (10^{-2} M) (b), and without additives (c).

of the spin probe with temperature is clearly visible. At 130 K, the powder pattern is observed; comparison of the powder pattern spectrum with that of free **T** at 130 K illustrates the reduction in the A_{zz} value upon complexation. This is due to the reduced polarity inside the CD cavity.

In order to extract the dynamic parameters for rotational motion of **T** inside the CD complex, we fitted the experimental spectra in Fig. 3 to the simulations using the software developed by Budil *et al.* on the basis of Liouville equation.[14] The spectra were simulated assuming a simple anisotropic rotational diffusion model with axial symmetry.† The long molecular axis was assumed to be parallel to the N–O bond. Although this approach does not reproduce the experimental lineshape perfectly, calculations using more complex models are highly inaccurate without multifrequency data, and are strongly model-dependent. We believe that our approach provides fairly accurate (albeit simplified) estimate of molecular motion. By fitting the simulated spectra to the experimental ones, we found the rotational diffusion rates $r_{\|}$ (parallel to the long molecular axis) and $r_{\perp}$ (perpendicular to the long molecular axis). In all simulations, the rotation across the long molecular axis was faster than rotation across the perpendicular axis, *e.g.*, $r_{\|} > r_{\perp}$. Activation energy (ΔE) for the rotation of the spin probe inside the cavity was evaluated from the diffusion rates $r_{||}$ and $r_{\perp}$ using Arrhenius equation (Fig. 4).

The activation energy was found to be 5.8 and 13.7 kJ mol^{-1} for the rotation across parallel and perpendicular axis, respectively. These values are quite low and would be consistent with the rotation of the label as a whole inside the cavity, with only small interactions with the cavity walls, particularly for the parallel mode. These data are also consistent with the recent literature on the rotation of spin probes in solid CD complexes (*vide infra*).

Competition with other guests

Vitrification could occur with poor homogeneity, and formation of aggregates or pools of fluid solutions trapped inside the vitreous phase is difficult to rule out. In order to unambiguously prove that the observed changes in the EPR spectra are related to the host–guest complexation and are not heterogeneity artefacts, we carried out competition experiments. Fig. 5 shows spectra of **T** recorded at 210 K in 8 : 2 (w/w) water : glycerol solution of β-CD (10^{-2} M) in the absence and in the presence of adamantanol. Adamantane derivatives are known to possess high affinity for β-CD,[15] and thus would be expected to effectively compete with **T**.

In the presence of the competing guest, the EPR spectrum of **T** : β-CD solution is indeed indistinguishable from that of uncomplexed **T**, confirming the displacement of **T** from the CD cavity by adamantanol.

Interestingly, replacement of β-CD with α-CD leads to EPR spectra in water : glycerol solutions at 210 K that are indistinguishable from the spectra of the free spin probes. This is consistent with the lack of strong binding between spin probes and α-CD (which has a smaller cavity than β-CD).

Physical mixtures and solid complexes of TEMPO spin probes with α-CD and β-CD

Very recently, several papers reported on the molecular dynamics of encapsulated nitroxides in supramolecular complexes in the solid state.[16–19] Dzikovski *et al.*[16] have described the mobility of DOXYL-labelled stearic acids and alkyl-derivatised TEMPO in solid β- and γ-CD complexes, using X-band and high frequency EPR. In these complexes, the alkane chain threads through the cyclodextrins and the nitroxide is located outside the CD cavity. Relatively fast rotation of the spin probes at room temperature was observed, and some evidence of restricted molecular motion was reported at very low temperatures (below 77 K). The barrier for rotation varied in the range 7–26 kJ mol^{-1} for different compounds. The lower values are only consistent with the rotation of the whole molecule inside the CD unit. Higher rotational barrier presumably indicates stronger interactions between the probe and the CD unit. Paramagnetic complexes of calixarenes with various nitroxides were also investigated in the solid state by EPR using multifrequency continuous wave[17–19] and echo-detected EPR, and single-crystal X-ray diffraction.[18,19] For these systems, a 1 : 2 nitroxide : calixarene stoichiometry was observed. The motion of the nitroxide inside the calixarene nanocapsules was again relatively fast at room temperature. Molecular motion was analysed assuming rapid jumps between restricted set of orientations.

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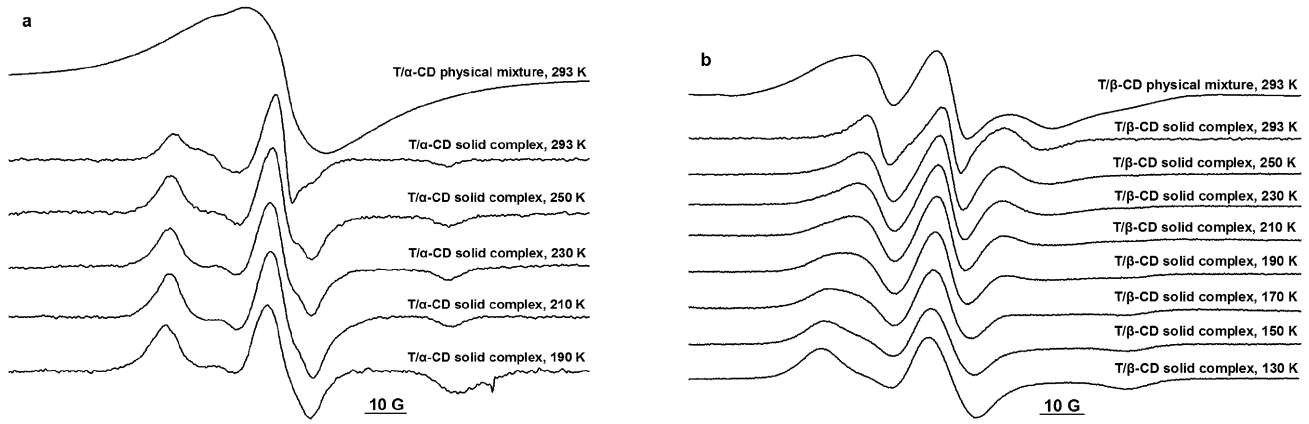

Fig. 6 EPR spectra of physical mixtures and solid complexes of **T** with α-CD (a) and β-CD (b).

In view of these results, we investigated solid complexes of **T** with α- and β-CD. The solid complexes were prepared by two methods. In the first approach, the spin probe and CD were ground together to prepare a physical mixture. The weight ratio of spin probe to the host was 1 : 100. In the second approach, the solution of the spin probe was mixed with the solution of CD, and the mixture was rotary evaporated at 60 °C. With the physical mixture of **T** with α-CD, only a broad line was observed in the EPR spectrum (Fig. 6a), suggesting the presence of bulk particles of **T**. The inclusion complex was thus not formed. The solid complex of α-CD prepared by solution evaporation showed immobilised **T** spectrum, with no sign of bulk segregation. The signal intensity was low, presumably due to the high volatility of **T** under evaporation conditions. The spectrum of this material showed little temperature dependence. The high volatility and lack of bulk segregation of **T** in the solid complex suggest that there might be only weak binding between **T** and α-CD; however the molecules of **T** in this composite have little rotational dynamics.

Complexes of β-CD with **T** showed different behaviour. With the physical mixture, a partially resolved nitroxide spectrum was observed which overlapped a broad line (Fig. 6b). This clearly shows that formation of inclusion complex can be achieved by simply grinding together the individual components in the solid state. The solid complex prepared by solution evaporation showed a broadened but resolved nitroxide spectrum. The broadening was clearly caused by the dipole–dipole interactions between the adjacent spin labels. The dipole–dipole interaction in X-band spectra is visible up to *ca.* 3 nm distance between neighbouring nitroxides. It is therefore likely that the **T** molecules encapsulated in adjacent CD moieties in the solid complex would exhibit noticeable dipole–dipole interactions. In order to minimise the effect of dipole–dipole interactions, we reduced the **T** : CD molar ratio to 1 : 2000. Interestingly, even at this low ratio, the EPR intensity of the evaporated solution remained strong, thus suggesting the low volatility of encapsulated **T**.

Fig. 6b shows the EPR spectra of the solid complex **T** : β-CD recorded at different temperatures. The spectra closely resemble those of vitrified solutions of the **T** : β-CD complex at the same temperature (Fig. 3). The a_N hyperfine values for the two systems were also nearly equivalent. This suggests that the CD unit effectively shields the nitroxide from the solvent, and the environment inside the complex does not depend on the presence of solvent outside the cavity. The solid complexes prepared by solvent evaporation are cyclodextrin hydrates. To completely remove water from the **T** : β-CD solid complex, it was kept overnight under vacuum at 100 °C. We found that the spectrum of the anhydrous complex was very similar to that of the hydrate,† further supporting the conclusion that the nitroxide unit is well shielded from the solvent by the CD cavity.

Conclusions

The results presented in this work show that in viscous solutions of host–guest complexes of TEMPO-based spin probes with cyclodextrins, the tumbling of the probe inside the cavity is faster than in free solution. This makes it possible to differentiate between free and encapsulated labels, which is a difficult task for room temperature spectra. Under these conditions, spin labels form 1 : 2 complexes with β-CD. Affinity of the spin probes to the CD cavity is influenced by the functional groups attached to the TEMPO moiety. For instance, binding constants for complexes of β-CD with **TOH**, **TNH$_2$** or **CT** are smaller than that for **T**. This is consistent with the earlier results which showed that functionalisation of TEMPO reduces the strength of binding with CDs.[10]

The tumbling rates of encapsulated probes are very similar to those in solid (*e.g.*, solvent-free) complexes. This suggests that rotational movement of the encapsulated spin probe is well shielded from the solvent. Small activation barrier for rotational diffusion is also consistent with this conclusion.

Experimental

Materials

The spin probes, TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy) derivatives, adamantanol, α- and β-CD were obtained from Sigma-Aldrich and used as received.

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Solution preparation

Stock solution for each spin probe was prepared in ethanol at 10^{-2} M concentration. To prepare the samples for EPR measurements, an aliquote of the ethanol solution was evaporated. The residue was then dissolved in 8 : 2 (w/w) water : glycerol mixture to make *ca.* 5×10^{-4} M solution. In the case of 4-amino-TEMPO (**TNH_2**) and 4-carboxy-TEMPO (**CT**), solvent mixtures were prepared using phosphate buffer in 8 : 2 (w/w) water : glycerol at pH 2 and 10. Concentration of β-CD was varied in the range 0–10^{-2} M. The EPR samples were prepared by mixing an appropriate amount of spin label and β-CD solutions. The solutions were then transferred to glass capillaries and sealed prior to recording EPR spectra.

Preparation of solid complexes

Physical mixtures of **T** and α-CD or β-CD were prepared by grinding the solid mixtures with pestle and mortar in a 1 : 100 mass ratio, until a homogeneous mixture was obtained in each case.

Solid complexes of **T** with β-CD and α-CD were prepared by rapid rotary evaporation of water from solutions containing CD and spin probe at 60 °C. The CD : **T** ratio was varied in the range 1 : 20 to 1 : 2000.

EPR measurements

The EPR spectra were recorded on a Bruker ESP-300E instrument at 9.76 GHz (X-band) at temperature in the range 130–250 K with 100 kHz modulation frequency, 0.998 mW microwave power, 320 s sweep time, 1 G modulation amplitude, and time constant 0.3 s. Samples were always cooled down from the room temperature to the desired temperature in the cavity. Before recording, all samples were allowed to equilibrate for at least 5 min after reaching the selected temperature.

Acknowledgements

Gabriela Ionita would like to acknowledge for research funding from CNCSIS—Romania PNII-Idei-46/2007.

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