An EPR method for measuring the rate of distribution of organic substrates between cyclodextrin, micelles and water†

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The combined use of selected nitroxides and EPR spectroscopy has been proved to be suitable for studying the partitioning rate of a given substrate in cyclodextrin–micelle systems.

Mixed organized media represent an intriguing system because of the possibility for a given guest to interact selectively with a single binding site.[1] When a substrate is added to such system the noncovalent binding of the guest (G) with the different hosts (H) to form a guest–host complex (C) is observed:

$$G + H \underset{k_{OFF}}{\overset{k_{ON}}{\rightleftharpoons}} C \qquad (1)$$

where k_{ON} and k_{OFF} are the rate constants of complex formation and dissociation, respectively. The stability of the complex is generally described in terms of the equilibrium dissociation constant, $K_d = k_{OFF}/k_{ON}$. In order to understand the chemistry of this system, it is vital to know the rate at which an organic molecule is partitioned between the different phases.[2]

One of the most studied system is the cyclodextrin–micelle mixed system, because of its application in separation-based affinity methods (*i.e.* capillary electrophoresis),[3] as a reaction medium[4] and in supramolecular devices.[5] While rates of solubilization into surfactant solutions[6] and of the inclusion complexation by cyclodextrins[7] (CDs) of specific probes have been extensively investigated using a variety of methods, the direct measure of individual binding events between analyte molecules and a mixture of both CD and micelle is still challenging.

EPR spectroscopy is characterised by a peculiar timescale which is comparable to that of many complexation processes. Here we introduce and describe an EPR method that allows, for the first time, direct and simultaneous measurement of the concentration of an organic spin probe in three different "pseudo-phases" (namely SDS micelles, CDs and water), if it exceeds the limit of detection. This new method allows the determination of all thermodynamic and kinetic parameters, K_d, k_{ON}, and k_{OFF}, in a single experiment that requires only a minute amount of substrates. This has the potential for significantly augmenting the arsenal of methods available for studying the partitioning of a given substrate in different pseudo-phases.

The method is based on the significant differences in the EPR parameters shown by *tert*-butyl benzyl nitroxide (TBBN) when it experiences water,[8] cyclodextrin cavity[9] or micellar environments[10] (see Table 1). The partitioning of nitroxide probes in the hydrophobic environment of SDS micelle gives rise to a reduction of the value of both nitrogen and β-protons splittings, with the result that the resonance fields for the $M_I(2H_\beta) = \pm 1$ lines of the free and included species are significantly different from those of the nitroxide dissolved in water. These differences are even more pronounced when TBBN is included in the cavity of CD's due to both polar and conformational changes occurring upon complexation.

The EPR spectra also show a strong linewidth dependence on temperature both in the presence of SDS micelle and CD, indicating that the lifetime of the radical in the associated and free form is comparable to the EPR timescale. Because of this favorable feature, the analysis of the line shape makes it possible to measure the rate constants for the partition of the probe in the pseudo-phases.

The EPR spectra at 298 K of TTBN, produced by reaction of the magnesium salt of monoperoxyphthalic acid with *tert*-butyl benzyl amine in the presence of heptakis-(2,6-*O*-dimethyl)-CD (DM-β-CD) 5.3 mM or SDS 33 mM are shown in Fig. 1a and 1b, respectively. The spectra show in both cases two sets of signals due to the radical in the aqueous phase and to that included in the CD cavity (Fig. 1a) or solubilised in the micellar pseudo-phase (Fig. 1b). In the presence of a mixture of CD and SDS at the same concentration as above, the EPR spectrum differs significantly from the previous ones (see ESI†). All the spectra can be correctly reproduced only by assuming the kinetic scheme reported in Scheme 1 in which the radical probe is exchanging, with a rate comparable to the EPR time scale, between the water phase and both the CD cavity and the micellar pseudo-phase.

Table 1 EPR parameters of *tert*-butyl benzyl nitroxide (1 G = 0.1 mT)

	$a(N)$/G	$a(2H_\beta)$/G	*g*-factor
Water	16.69	10.57	2.0056
SDS	16.04	8.84	2.0057
DM-β-CD	15.60	7.80	2.0058

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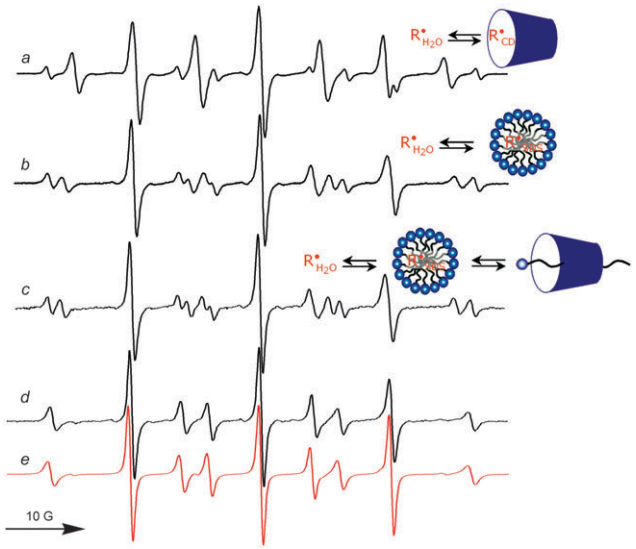


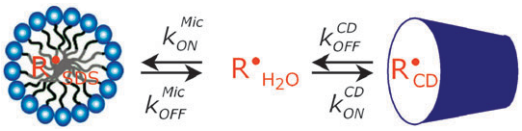
Fig. 1 EPR spectra of TBBN recorded in water at 298 K. (a) DM-β-CD 5.3 mM; (b) SDS 33 mM; (c) β-CD 16 mM and SDS 49 mM; (d) DM-β-CD 16 mM and SDS 49 mM. (e) Theoretical simulation of spectrum (d) obtained with the rate constant reported in Table 1, entry 11.

Simulation of the exchange-broadened EPR spectra, by using well established procedures based on the density matrix theory[11] and assuming a three-jump model as illustrated in Scheme 1, led to the determination of the residence time (τ_X) of the paramagnetic species in the three different pseudo-phases which are related to rate constants (see Table 2) of the exchange processes by the following expressions:

$$\tau_{CD} = 1/k_{OFF}^{CD};\ \tau_{MIC} = 1/k_{OFF}^{MIC};\ \tau_{H_2O} = 1/k_{ON}^{CD} + 1/k_{ON}^{MIC} \quad (2)$$

By recording the corresponding EPR spectra it is possible, therefore, to obtain the distribution of the radical probe in the different environments at different concentrations of surfactant and cyclodextrin. As an example, in Fig. 2 is reported the variation of the molar fraction of TBBN partitioned in the micellar phase in the presence of different DM-β-CD/SDS concentrations.

Depending on the relative amount of CD and SDS we can distinguish different regimes:



Scheme 1

[SDS] ≤ [CD]: Under this condition the amount of radical included in the CD cavity decreases proportionally to the amount of surfactant present in the solution. This effect is attributed to complexation of the surfactant monomer by CD and release of the probe into the bulk aqueous medium giving rise to an increase in the residence time of the probe in the water phase (entries 3–4). According to the fact that in this condition the free dissolved SDS monomer concentration is well below the critical micelle concentration (cmc) no EPR signals due to the radical partitioned in the SDS phase are observed.

[SDS] > [CD]: The formation of SDS–CD complex increases the concentration of surfactant required for micellization,[12] and the critical micelle concentration of a micellar system in the presence of a cyclodextrin (cmc_{app}) is equivalent to the combined concentrations of surfactant monomers complexed to the CD, and of free dissolved monomer in equilibrium with the micellized surfactant, cmc_{real}. Once micellization starts, the system behaves like a typical micellar system. Analysis of EPR data shows that this is actually the case when β-CD (see Entries 5–8) is employed as the macrocyclic host. As an example Fig. 1c shows the EPR spectrum recorded in the presence of SDS 49 mM and β-CD 16 mM which perfectly matches that one recorded in the presence of SDS 33 mM (Fig. 1b).

A change in the nature of the macrocyclic host has, however, a dramatic effect on the partitioning behaviour of the radical probe. In the presence of a small amount of methylated cyclodextrins (DM-β-CD or TM-β-CD) the EPR spectra are characterised by an increased broadening of the external lines, indicating that the exchange rate of the probe between water and the micellar pseudo-phase is becoming faster (see entries 9–10). Further addition of methylated cyclodextrins in the solution results in a dramatic decrease of the life time of the probe in the SDS micelles. Nevertheless, when the

Table 2 Selected EPR rate constants at 298 K for the partition of TBBN in the micellar and CD locations

Entry	[SDS]/mM	Cyclodextrin (mM)	k_{ON}^{CD}/s^{-1}	k_{OFF}^{CD}/s^{-1}	k_{ON}^{MIC}/s^{-1}	k_{OFF}^{MIC}/s^{-1}
1	—	β-CD (5.3)	3.6×10^6	5.3×10^5	—	—
2	—	DM-β-CD (5.3)	3.4×10^6	6.1×10^5	—	—
3	3.4	DM-β-CD (5.3)	1.7×10^6	6.5×10^5	—	—
4	5.0	DM-β-CD (5.3)	3.4×10^5	7.0×10^5	—	—
5	33	—	—	—	6.2×10^6	3.9×10^6
6	26	β-CD (16)	6×10^4	5.3×10^5 [a]	8.0×10^5	3.9×10^6
7	33	β-CD (16)	$<3 \times 10^4$	5.3×10^5 [a]	3.0×10^6	3.9×10^6
8	49	β-CD (16)	$<3 \times 10^4$	5.3×10^5 [a]	6.2×10^6	3.9×10^6
9	33	DM-β-CD (5.3)	$<4 \times 10^4$	6.1×10^5 [b]	5.5×10^6	7.8×10^6
10	33	DM-β-CD (20)	8.0×10^4	6.1×10^5 [b]	1.6×10^7	4.7×10^7
11	49	DM-β-CD (16)	4.0×10^4	6.1×10^5 [b]	1.5×10^7	2.8×10^7
12	105	DM-β-CD (20)	$\ll 4 \times 10^4$	6.1×10^5 [b]	1.5×10^7	1.0×10^7
13	73	DM-β-CD (40)	8.0×10^4	6.1×10^5 [b]	2.2×10^7	2.8×10^7
14	53	TM-β-CD (20)	—	—	1.5×10^7	2.4×10^7

[a] Assumed equal to that of entry 1. [b] Assumed equal to that of entry 2.

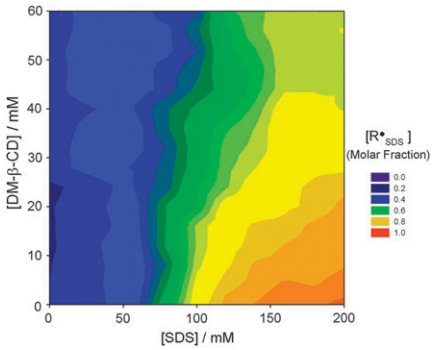


Fig. 2 Molar fraction of TBBN partitioned in SDS micelles in the presence of different DM-β-CD/SDS concentrations.

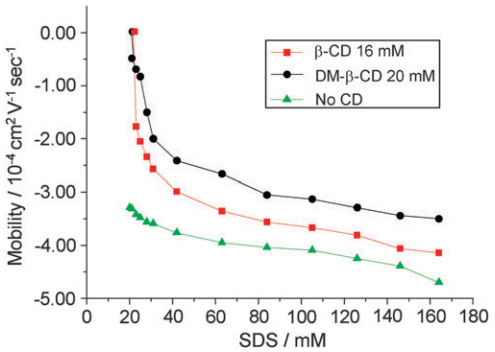


Fig. 3 Mobility data of *tert*-butyl benzyl ketone as a function of the SDS concentration and the nature of cyclodextrin employed.

concentration of DM-β-CD is so much so that ([CD] + cmc_{real}) > [SDS], the EPR spectrum can be correctly simulated by admitting that a sizeable fraction of the probe is still experiencing a hydrophobic environment. While the dramatic reduction of the residence time of the radical guest in the micelle suggests that the micellar structure is altered significantly in the presence of methylated cyclodextrins, the existence of radicals dissolved in an hydrophobic aggregate for concentration of SDS below cmc_{app} (cmc_{app} = cmc_{real} + CD) indicate that this altered micelle is still able to solubilise the probe. According to recent findings by Dreiss and coworkers[13] we can suppose that the presence of methylated cyclodextrins results in a lowering of the aggregation number, and in an increase of solvent penetration and polydispersivity of the micelle.

From an applicative point of view we checked if the residence time of the probe in each environment determined by EPR could be employed to predict the electrophoretic behaviour of a given analyte. Capillary electrophoretic (CE) analysis of neutral solutes in CD-micellar systems is based on the differences in the mobility of the analytes in the homogeneous phase. The anionic SDS micelle acts as a carrier and the inclusion of the solutes into the neutral CD cavity is a process in competition with the partitioning into the micelle. When the residence time of the probe in the cyclodextrin is long compared with the residence time in the micelle, the mobility of the neutral probe approaches to zero; this condition is also observed when surfactant concentration is below the critical micelle concentration.

CE experiments were performed by analyzing the electrophoretic behaviour of *tert*-butyl benzyl ketone, the diamagnetic analogue of TBBN, in the presence of either β-CD and DM-β-CD at 16 and 20 mM, respectively. SDS was supplemented as a micelle separation carrier in a wide concentration range (15–165 mM) and the effective electrophoretic mobility (μ_e) of the probe was plotted against the SDS concentration (Fig. 3).

As expected, in the absence of cyclodextrin only small mobility variations were observed in the investigated SDS range, while, in the presence of β-CD, the mobility variation profile shows a marked break point which corresponds to a surfactant concentration below cmc_{app} ([β-CD] + cmc_{real} = 24 mM). In the presence of DM-β-CD the effective mobility of the carbonyl probe is significantly reduced. According to EPR data this should be attributed to the reduced residence time of the probe in the micellar pseudo-phase. Conversely to that found with β-CD, in the presence of SDS between 20 and 28 mM, that is below the hypothetical cmc_{app} ([DM-β-CD] + cmc_{real} = 28 mM) the transport ability of the micellar carrier is still maintained with DM-β-CD, this being an indication that a modified micellar carrier is present in the solution as predicted by EPR measurements. Work is in progress to investigate the effect of methylated CDs on the CE-based separation of enantiomers when the SDS concentration is in the premicellar range.

In conclusion, the combined use of selected nitroxide and EPR spectroscopy has been proved to be suitable for studying the partitioning rate of a given substrate in CD–micelle systems. On the condition that the spectroscopic parameters of the probe are sufficiently different to distinguish the different environment experienced by the radical, EPR data can be employed to predict the partitioning behaviour of non radical analytes in mixed organised systems. We foresee the potential role of EPR in extending the utility of this technique by using probes characterized by a different lipophilicity[14] or containing a chiral centre.[15]

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