Spin-trapping of free radicals by PBN-type β-phosphorylated nitrones in the presence of SDS micelles †

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Three PBN-type β-phosphorylated nitrones, PPN 1, 4-PyOPN 2, and 4-ClPPN 3, have been used to trap two carbon centred free radicals, $CH_3^{\bullet}$ and $CO_2^{\bullet -}$, in a water–SDS biphasic system. The location of the traps and their adducts has been found by EPR spectroscopy. 4-PyOPN is found to remain in water, while 4-ClPPN is preferentially sequestered into the micellar structure, and PPN partitioned between the two phases. With the three nitrones, the $CO_2^{\bullet -}$ spin adduct always remains in the bulk aqueous phase. In contrast, the three $CH_3^{\bullet}$ spin adducts seem to be present in both phases. However, 4-PyOPN-CH_3 is essentially located in water, and 4-ClPPN–CH_3 in the micelles, while PPN–CH_3 is clearly shown to partition between the aqueous environment and the micellar interior. For all these spin adducts, the phosporus hyperfine splitting constant, a_P, is found to be a good indicator of aminoxyl behaviour in the presence of micelles. For the various sodium dodecyl sulfate concentrations, the PPN–CH_3 affinity for the micellar phase was evaluated from the average a_P value.

The use of nitrones for the *in vivo* detection of free radicals is of growing importance, since radicals have been shown to be involved in the development of many pathological conditions.[1–3] To be an efficient tool in the study of radical processes occurring in biological cells, a spin trap must fulfil three main conditions. First, it has to react rapidly with free radicals, giving rise to rather persistent spin adducts in polar environments. Secondly, the EPR spectra of the various spin adducts must be characteristic of the radical trapped. Finally, it has to be hydrophilic enough to be used in biological media but also lipophilic enough to cross biomembranes to enter the cells.

Among all the commercially available nitrones, DMPO (5,5-dimethyl-3,4-dihydropyrrole *N*-oxide) and PBN (*N*-*tert*-butylbenzylideneamine *N*-oxide) remain the most popular,[4,5] but their use in the *in vivo* identification of free radicals is not without its limitations. For example, DMPO has been shown to trap efficiently hydroxyl radical ($HO^{\bullet}$) in every kind of media, but its use in the detection of superoxide in water is dramatically limited by the short life-time of the corresponding spin adduct.[6,7] A few years ago, the synthesis of a new β-phosphorylated cyclic nitrone, DEPMPO (5-diethoxyphosphoryl-5-methyl-3,4-dihydropyrrole *N*-oxide), was described, and this compound was found to trap very efficiently superoxide in aqueous media, leading to an exceptionally persistent spin adduct.[8–10] But this trap was found to be as hydrophilic as its non-phosphorylated analogue, *i.e.* DMPO,[9,11] and this limits *in vivo* applications to extracellular media. In contrast, PBN and structurally related nitrones show various degrees of lipophilicity, depending on the nature of the substituents on the aromatic ring.[11–13] However, their *in vivo* spin trapping applications are limited to carbon-centred radical detection, since the corresponding oxyl-radical adducts decompose too rapidly in polar media.[14–18] One should also consider that the various adducts of PBN or its analogues often show similar EPR spectra, which could be a source of dramatic misinterpretations in spin trapping experiments.[16–21] So new and more lipophilic spin traps are still needed for the *in vivo* identification of various free radicals, including oxyl-radicals, and for the study of intracellular processes involving radical intermediates.

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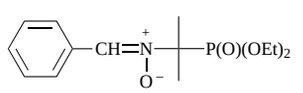
Recently, we described the synthesis of three β-phosphorylated nitrones derived from PBN:[22,23] PPN **1** (*N*-benzylidene-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide), 4-PyOPN **2** {1-diethoxyphosphoryl-1-methyl-*N*-[(1-oxidopyridin-1-ium-4-yl)methylidene]ethylamine *N*-oxide}, and 4-ClPPN **3** {*N*-[(4-chlorophenyl)methylidene]-1-diethoxyphosphoryl-1-methyl ethylamine *N*-oxide}.



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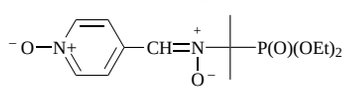

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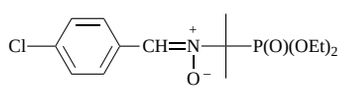

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These new compounds have been shown to trap efficiently not only carbon-centred radicals, but also superoxide even in polar environments, yielding reasonably persistent superoxide spin adducts in phosphate buffers.[24] In addition, these three compounds represent a relatively large scale of lipophilicity: the PPN octanol–water partition coefficient K_p has been evaluated at 10.1, indicating the same lipophilicity as PBN, while 4-PyOPN ($K_p = 0.18$) was found to be more hydrophilic and 4-ClPPN ($K_p = 195$) poorly water-soluble.[24] However, these traps have never been used *in vivo* and at the moment no information is available concerning their behaviour in the presence of biological cells.

The purpose of this work was then to predict the possible capacity of PPN, 4-PyOPN and 4-ClPPN to cross the phospholipid bilayer of biomembranes. We have undertaken a spin-trapping study with these three nitrones in the presence of sodium dodecyl sulfate (SDS) micelles, considered here as a very simple membrane model. Two carbon-centred radicals, $CO_2^{\bullet -}$ and $CH_3^{\bullet}$, have been generated in the bulk aqueous phase at various SDS concentrations in the presence of a nitrone, by carrying out a standard Fenton reaction in the pres-

Table 1 EPR parameters of the various $CO_2^{\bullet -}$ radical spin adducts in pure water

Spin adduct	a_N/mT	a_H/mT	a_P/mT
PPN–CO_2^-	1.44	0.45	4.97
4-PyOPN–CO_2^-	1.41	0.29	4.84
4-ClPPN–CO_2^-	1.45	0.42	5.00

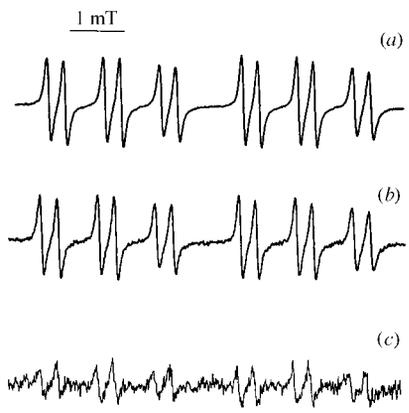


Fig. 1 EPR signal of 4-ClPPN–CO_2^- recorded at various SDS concentrations: (*a*) [SDS] = 0 mol dm^{-3} (receiver gain, 12 000), (*b*) [SDS] = 10 mmol dm^{-3} (receiver gain, 20 000), and (*c*) [SDS] = 50 mmol dm^{-3} (receiver gain, 63 000)

ence of sodium formate or dimethyl sulfoxide, respectively. The location of the three nitrones and of their spin-adducts has been determined from the detailed analysis of the various spin adduct EPR spectra recorded in the presence of SDS micelles.

Results and discussion

Whatever the environment polarity the three nitrones employed, *i.e.* PPN, 4-PyOPN and 4-ClPPN, have been shown to give persistent spin adducts with various carbon-centred free radicals,[23] and were used in this study to trap $CH_3^{\bullet}$ and $CO_2^{\bullet -}$ at different SDS concentrations.

$CO_2^{\bullet -}$ trapping

First, we examined the EPR signals obtained by trapping $CO_2^{\bullet -}$ with each one of the three nitrones. In the absence of SDS, the corresponding spin adducts, *i.e.* PPN-CO_2^-, 4-PyOPN-CO_2^- and 4-ClPPN-CO_2^-, presented the hyperfine splitting constants (hfscs) given in Table 1.

All these spectra remained unchanged when the SDS concentration was kept lower than the critical micellar concentration (CMC), which is *ca.* 8.2 mmol dm^{-3}.[25] At higher SDS concentration, in the presence of micelles, different behaviour was observed with the three traps. Using 4-PyOPN as spin trap, both the intensity and the hfsc values determined on the 4-PyOPN-CO_2^- spectra remained the same, whatever the SDS concentration. This clearly shows that, in the presence of micelles, both 4-PyOPN and its $CO_2^{\bullet -}$ spin adduct were located in the bulk aqueous phase. In contrast, when 4-ClPPN was used as spin trapping agent, an increase in the SDS concentration resulted in a strong decrease in the 4-ClPPN–CO_2^- EPR signal intensity and, at 50 mmol dm^{-3} SDS, this spectrum almost vanished (see Fig. 1). These results indicate that the nitrone 4-ClPPN shows a strong affinity for the micellar phase, although the corresponding spin adduct was always located in the aqueous environment.

The nitrone PPN represented an intermediate between these two extreme cases. When the SDS concentration was raised above the CMC, a significant decrease in the PPN–CO_2^- EPR signal was detected, while the hfsc values remained unchanged, but this signal was always clearly observed at high SDS concentrations, such as 100 mmol dm^{-3}. This indicates that the nitrone PPN was partitioned between the micellar environment and the bulk aqueous phase, although the corresponding spin adduct was unable to penetrate the micelles.

Table 2 EPR parameters of the various $CH_3^{\bullet}$ spin adducts in pure water and in micelles, determined by EPR signal simulation

	hfsc values in water/mT			hfsc values in micelles/mT		
Nitroxide	a_N	a_H	a_P	a_{Nm}	a_{Hm}	a_{Pm}
4-PyOPN–CH_3	1.45	0.24	4.67	1.43	0.16	4.15
PPN–CH_3	1.49	0.35	4.66	1.39	0.40	4.39
4-ClPPN–CH_3	1.49	0.32	4.64	1.30	0.30	4.45

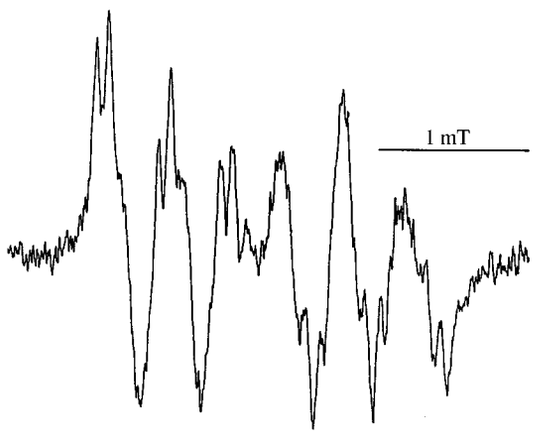


Fig. 2 EPR signal recorded by carrying out a standard Fenton reaction in the presence of 0.2 mol dm^{-3} sodium formate, 0.05 mol dm^{-3} 4-PyOPN and 0.1 mol dm^{-3} SDS. The signal obtained was found to correspond to the superposition of two nitroxide spectra, one being the $CO_2^{\bullet -}$ radical spin adduct of 4-PyOPN ($a_N = 1.41$, $a_H = 0.29$ and $a_P = 4.84$ mT), the other being probably the 4-PyOPN spin adduct of a carbon-centred radical derived from SDS monomers ($a_N = 1.40$ and $a_P = 3.83$ mT).

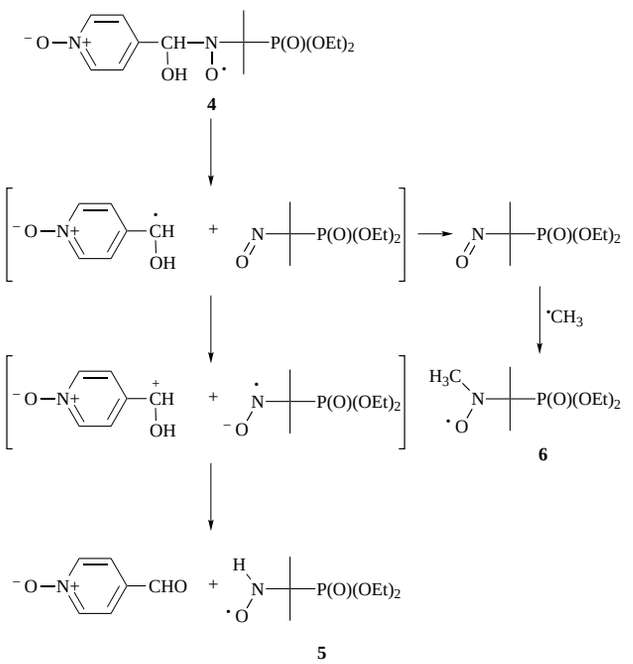
It should be mentioned here that, at high SDS concentration (*ca.* 100 mmol dm^{-3}), a second paramagnetic species was always observed when trapping $CO_2^{\bullet -}$ with both 4-PyOPN and PPN (see Fig. 2). This radical EPR spectrum exhibited six broad lines, and showed the following hfsc values: $a_N = 1.40$ and $a_P = 3.83$ with 4-PyOPN, $a_N = 1.46$ and $a_P = 3.92$ mT with PPN. The same signals were recorded when $HO^{\bullet}$ was generated by a standard Fenton system in the presence of 100 mmol dm^{-3} SDS and in the absence of sodium formate, *i.e.* in the absence of $CO_2^{\bullet -}$. The same kind of phenomenon has previously been observed by Bakalic and Thomas[26] who noticed the formation of a carbon-centred radical adduct of a nitroso spin trap when $HO^{\bullet}$ was generated in the presence of SDS at high concentration. According to their results and to our observations, the signals recorded in our experiments with both 4-PyOPN and PPN could be reasonably assigned to the spin adducts resulting from the addition to these nitrones of a radical produced by $HO^{\bullet}$ attack of the SDS monomers solubilised in the bulk aqueous phase. Because of the large linewidth, which was always 0.3–0.5 mT, it was impossible to resolve the coupling with the β-hydrogen.

$CH_3^{\bullet}$ trapping

The trapping of the methyl radical by 4-PyOPN in the absence of SDS yielded the corresponding spin adduct, 4-PyOPN–CH_3 ($a_N = 1.45$, $a_H = 0.24$ and $a_P = 4.67$ mT). No change in the spectrum was observed while the SDS concentration was raised to *ca.* 20 mmol dm^{-3}. Above this value, a second minor paramagnetic species was detected that showed the following hfsc values: $a_N = 1.43$, $a_H = 0.16$, and $a_P = 4.15$ mT (see Table 2). Since this signal was found to grow while the SDS concen-

tration was increased, we thought that it might correspond to the 4-PyOPN–CH_3 spin adduct in the micellar phase.

Whatever the SDS concentration, two other radical species were also observed by EPR spectroscopy. The first one showed the following hfsc values: $a_N = 1.31$, $a_H = 1.26$ and $a_P = 5.20$ mT, and has previously been identified as the aminoxyl **5**.[22] The second one ($a_N = 1.57$, $a_H = 1.33$ (3H) and $a_P = 5.10$ mT) corresponds to nitroxide **6**. According to the mechanism proposed for the decay of hydroxyl radical spin adducts of PBN-type nitrones[16] and to previous results,[22] both compounds **5** and **6** were believed to be formed from the decomposition of the hydroxyl radical spin adduct of 4-PyOPN (4-PyOPN-OH, **4**), as shown in Scheme 1.



Scheme 1 Decomposition pathway of the hydroxyl radical spin adduct of 4-PyOPN (4-PyOPN–OH, **4**) in aqueous medium in the presence of methyl radical

When the methyl radical was trapped by PPN in water in the absence of SDS, the EPR spectrum of the corresponding spin adduct (PPN–CH_3) showed the hfsc values given in Table 2. This signal remained unchanged as long as the SDS concentration was kept below the CMC. From *ca.* 8 mmol dm^{-3} SDS, significant variations were observed in a_N and a_P values, while only a slight increase was detected for a_H. Thus, a_N and a_P lost 0.05 and 0.15 mT, respectively, while the SDS concentration was increased from 1 to 100 mmol dm^{-3} (see Fig. 3). The a_N decrease indicated a diminution in the polarity of the spin adduct environment, and the a_P decrease could also indicate a modification in the nitroxide conformation. These two results were both in favour of a partial penetration of PPN–CH_3 into the micellar phase. The a_N and a_P values obtained for different SDS concentrations correspond to average values of the hfsc of the aminoxyl in water and in the micelle.

From this average value found for the coupling with the phosphorus, denoted $\langle a_P \rangle$, the affinity of the aminoxyl PPN–CH_3 for the micellar phase could be estimated. An expression for $\langle a_P \rangle$ is given by eqn. (1), in which a_{Pm} and a_{Pw} represent a_P

$$\langle a_P \rangle = (a_{Pm} - a_{Pw})x_m + a_{Pw} \quad (1)$$

values in the micellar phase and in water, respectively, x_m being the molar ratio of the aminoxyl present in the micelles. The aminoxyl affinity for the micellar phase could be evaluated by a distribution coefficient K_d, defined as the ratio of aminoxyl moles in micelles divided by the number of SDS moles associated in micelles, to the aminoxyl moles in water divided by the water mole number, as indicated by eqn. (2), in which n_{Nm} and

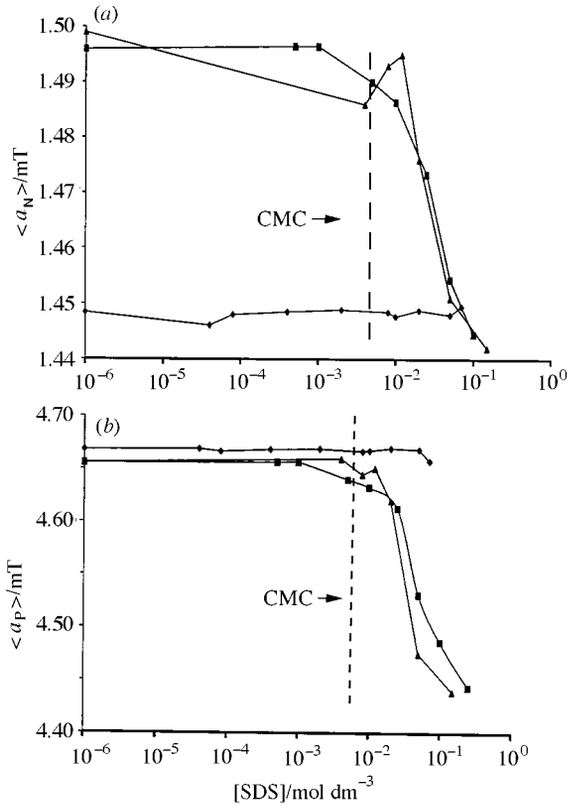


Fig. 3 Experimental variation of average values of (*a*) a_N and (*b*) a_P of PPN–CH_3 (■), 4-PyOPN–CH_3(◆) and of 4-ClPPN–CH_3 (▲) *versus* SDS concentration (logarithmic scale)

$$K_d = \frac{n_{Nm}/n_{SDS}}{n_{Nw}/n_w} \quad (2)$$

n_{Nw} represent the number of aminoxyl moles solubilised in micelles and in water, respectively, n_{SDS} being the number of SDS moles associated in micelles and n_w the number of water moles in the medium. Eqn. (3) was then derived from eqn. (2).

$$K_d = \frac{x_m n_w}{(1 - x_m)n_{SDS}} \quad (3)$$

Eqn. (1) and (3) led to eqn. (4).

$$\langle a_P \rangle = \frac{(a_{Pm} - a_{Pw})K_d}{K_d + n_w/n_{SDS}} + a_{Pw} \quad (4)$$

This distribution coefficient K_d should be considered as a good indicator of the aminoxyl affinity for the micellar phase. In addition, K_d was directly related to the micelle–water partition coefficient K_P of the aminoxyl considered, which can be defined as the ratio of the aminoxyl concentration in micelles to that in water. Thus, the relation between K_d and K_P is given by eqn. (5), in which V_w and V_m represent the total volume of

$$K_P = K_d \frac{n_{SDS} V_w}{n_w V_m} \quad (5)$$

water and micellar phases, respectively. Since the mean number of monomers in a micelle and the average radius of an SDS micelle have been reported to be 64 and *ca.* 25 Å, respectively,[25] V_m can be calculated approximately from the total number of SDS moles associated in micelles. Thus, eqn. (5) can be transformed into eqn. (6), which permitted us to evaluate K_P. In

Table 3 Determination of the hfsc values with the phosphorus and the nitrogen nuclei for PPN–CH_3 in micelle, and of their distribution (K_d) and partition coefficients (K_P) between water and micelle phases. These evaluations have been carried out using computer modelling of the variation observed in the mean hfsc values *versus* SDS concentration, using eqn. (4).

a_{Nm}/mT	a_{Pm}/mT	K_d	K_P
1.44 ± 0.1	4.40 ± 0.015	1270 ± 220	38.1

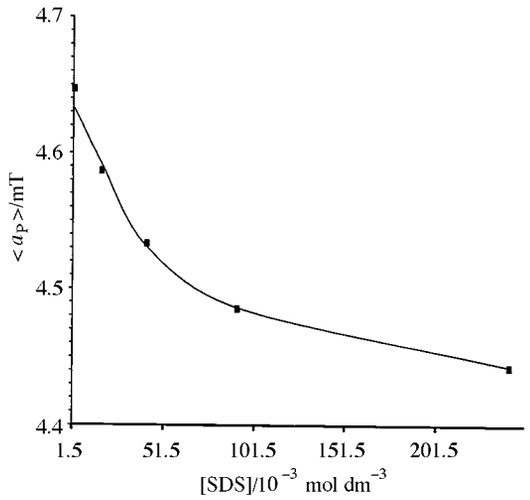


Fig. 4 Modelling of the $\langle a_P \rangle$ variation *versus* the concentration of SDS monomers associated in micelles (*i.e.* [SDS] − CMC), using eqn. (4). The hfsc a_P for the nitroxide in water has been determined on an EPR spectrum recorded in a pure water environment. The modelling led to the following parameters: a_{Pm} = 4.40 mT and K_d = 1270.

$$K_P \approx 0.03\ K_d \qquad (6)$$

Fig. 4, the $\langle a_P \rangle$ diminution observed in our experiments has been plotted against the concentration of SDS monomers associated in micelles, and computer modelling of this decrease has been achieved using eqn. (4). The good fit between experimental points and the calculated curve confirmed the validity of the method used to evaluate the affinity of the aminoxyl PPN–CH_3 for the micellar phase.

This computer modelling led to the determination not only of K_d, but also of the coupling constant with the phosphorus in a micellar environment, *i.e.* a_{Pm}. We thought that K_d was estimated much more precisely than K_P, since the evaluation of the total micellar volume was rather approximate. However, K_P could be considered as a more traditional indicator of a compound affinity for a lipophilic phase, and this is the reason why the values obtained for both K_d and K_P have been reported in Table 3, together with the hfsc values of PPN–CH_3 in micelle determined by two methods. The values thus determined for these two parameters (K_d = 1270 and K_p = 38.1) indicate that PPN–CH_3 shows a rather high affinity for the micellar phase. When the same method was applied to evaluate K_d from the variation of the average value of a_N, using eqn. (4) in which a_P has to be replaced by a_N, this led to an evaluation of a_N in the micellar environment (*i.e.* a_{Nm}, see Table 3). But in this case, the experimental decrease measured in $\langle a_N \rangle$ was much weaker, thereby making the evaluation of K_d less accurate. The existence of a strong coupling with the phosphorus, which permitted us to correctly evaluate the affinity of PPN–CH_3, for the micellar phase, should thus be regarded as an important advantage of the β-phosphorylated spin adducts.

In other respects, one should also notice that it was impossible to correctly simulate the PPN–CH_3 EPR signals recorded in the presence of micelles without taking into account an exchange of this adduct between the micellar and the water phases. For example, the PPN–CH_3 EPR signal shown in Fig. 5

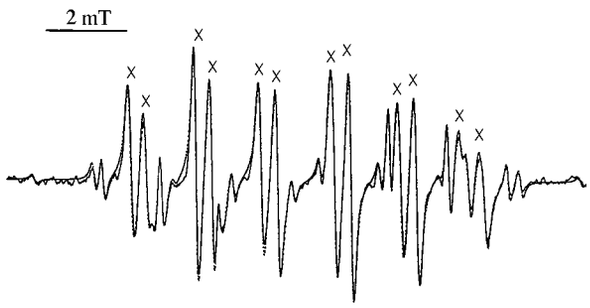


Fig. 5 Experimental EPR spectrum of PPN–CH_3 (——) recorded in the presence of 25 mmol dm^{-3} SDS and simulation of this signal (· · · ·) obtained by considering two paramagnetic species in rapid equilibrium, the first one (69%) being PPN–CH_3 in water (a_N = 1.49, a_H = 0.35, and a_P = 4.66 mT), the second one (31%) being the same spin adduct in micelles (a_N = 1.39, a_H = 0.40, and a_P = 4.39 mT). The exchange correlation time has been evaluated as 0.5×10^{-7} s. The lines which are not topped by a cross correspond to paramagnetic byproducts **5** and **6**.

was recorded in the presence of 25 mmol dm^{-3} SDS. The calculated spectrum, which has been superimposed on the experimental one, was obtained by considering an equilibrium between two adduct forms, one present at 69% corresponding to the aminoxyl solubilised in the bulk aqueous phase, and the other present at 31% (a_N = 1.39, a_H = 0.40 and a_P = 4.39 mT) corresponding to the same aminoxyl located into the micelles. The correlation time of the exchange was found to be *ca.* 0.5×10^{-7} s, which is in the range of the life-time of an SDS monomer in the micellar structure.[25]

It is important to notice here that almost the same values have been determined for the hfsc with the phosphorus nucleus for the aminoxyl in micelles by computer modelling of the average value of these coupling constants using eqn. (4) and by computer simulation of the EPR signal. The good agreement between these two methods confirms that PPN–CH_3 partitioned between water and micellar phases, and corroborates the validity of the evaluation of the spin adduct affinity for the micellar phase using K_d or K_P, determined from the variation observed in $\langle a_P \rangle$. In contrast, these two methods gave rather different results for a_N in micelles, and this is because the variation in the average constant $\langle a_N \rangle$ observed at the various SDS concentrations is too weak to allow a valid determination of this parameter in micelles.

By trapping $CH_3^{\bullet}$ with 4-ClPPN in the absence of SDS, the corresponding spin adduct (4-ClPPN–CH_3) was observed by EPR in pure aqueous media and its spectrum showed the hfsc values given in Table 2. This spectrum remained unchanged when the SDS concentration was kept below the CMC. However, in the presence of micelles, a significant diminution was observed in a_N and a_P values while SDS concentration was raised. Thus, a_N and a_P were found to diminish by 0.5 and 2 mT, respectively, while SDS concentration was increased from 1 to 150 mmol dm^{-3} (see Fig. 3). In addition, an important broadening of the lines was observed in the EPR spectra recorded in the presence of micelles, and this could be due to an immobilisation of 4-ClPPN–CH_3 in the micellar structure. But other causes, such as a second exchange of the aminoxyl between the micellar interface and the micellar interior, or the modification of the micelle shape at high SDS concentration, resulting in a diminution of the aminoxyl environment isotropy, could also induce the same kind of phenomenon. Nevertheless, all these results indicated that 4-ClPPN–CH_3 was preferentially located in the micellar phase, where its EPR spectrum exhibited the hfsc values indicated in Table 2.

Using the method previously described for PPN–CH_3, we tried to evaluate the 4-ClPPN–CH_3 affinity for the micellar phase by calculating first its K_d from eqn. (4), and then its K_P from eqn. (5). However in this case, the determination of these two parameters was too approximate, since the measurement of the average hfsc values was rather difficult because of the

broadening of the EPR lines. Thus, the method used to evaluate the two coefficients K_d and K_P indicated above cannot be applied when the aminoxyl considered is immobilised in the micellar structure.

Last, it is also important to notice here that the 4-ClPPN–CH_3 EPR signal was still observed in the presence of 150 mmol dm^{-3} SDS, while 4-ClPPN–CO_2^- was difficult to detect at 50 mmol dm^{-3} SDS. The difference between these two results could be explained by the following hypothesis. 4-ClPPN could be sequestered within the micellar structure by locating its polar group, *i.e.* the nitrone function, between the polar head groups of the SDS molecules, and by burying its aromatic moiety in the hydrophobic micelle interior. Thus, the methyl radical could be trapped at the water–micelle interface, while $CO_2^{\bullet -}$ could not approach the nitrone function because of electrostatic repulsions with the negatively charged micelle surface. Although this hypothesis is consistent with the results described by either Janzen *et al.*[27] or by Struhl *et al.*[28] in their studies concerning the location of hydrophobic traps in model membranes, it should also be considered that the dimethyl sulfoxide present in the reaction medium could enhance either the micelle permeability to the methyl radical or the nitrone solubility in the water phase.

Conclusion

This EPR spin trapping study allowed us to determine the localisation of the three nitrones PPN, 4-PyOPN and 4-ClPPN, and of their $CO_2^{\bullet -}$ and $CH_3^{\bullet}$ spin adducts in water–SDS micelle heterogeneous media. The hydrophilic 4-PyOPN was located in the aqueous phase, while the lipophilic 4-ClPPN was preferentially sequestered into the micellar structure. The nitrone PPN represents an intermediate between these two extreme cases, since it was found to partition between the micelles and the bulk aqueous phase.

When $CO_2^{\bullet -}$ was trapped with each one of the three nitrones, the corresponding spin adduct could never enter the micelles, and this is certainly due to the repulsive electrostatic forces between the negatively charged aminoxyls and the sulfate groups of SDS molecules.

In contrast, the methyl radical adducts of the nitrones always seemed to be present in both phases. However, in the case of 4-PyOPN–CH_3, the penetration of a small amount of this adduct into the micelles was uncertain, since all the spectra recorded for the various SDS concentrations have been correctly simulated considering the presence of two aminoxyls, but without taking into account a chemical exchange. In the case of PPN–CH_3 and of 4-ClPPN–CH_3, the existence of a rapid exchange of the aminoxyls between the two phases has been clearly and unambiguously demonstrated by the decrease observed in the hfsc values while SDS concentration was increased. As far as we know, regular variations of hfsc values in spin trapping studies performed in the presence of micelles[26,29–31] have never previously been observed. In the present case, such an observation was possible and easy because of the existence in these spin adducts of a strong coupling constant with the phosphorus nucleus, and this should be considered as an important advantage of our new spin trapping agents when comparing them to commercially available PBN-type nitrones. In particular, the a_P variation permitted us to evaluate for the first time the partition coefficient K_p of PPN–CH_3 between the bulk aqueous phase and the micelles. Since the evaluation of K_P depended on an approximation of the total volume of the micellar phase, we proposed the use of another coefficient K_d, the determination of which is much more precise, to quantify the aminoxyl affinity for the micellar phase. Thus, PPN–CH_3 (K_d = 1270) was found to partition significantly between the two phases. This determination of K_d, and of K_P, based on EPR measurements should be regarded as a new method for evaluating spin adduct lipophilicity, and experiments are planned in our laboratory to extend this technique to various stable β-phosphorylated aminoxyls. Nevertheless, this method is not usable when the aminoxyl considered shows too high an affinity for either the bulk aqueous phase (*e.g.* 4-PyOPN–CH_3) or for the micellar structure (*e.g.* 4-ClPPN–CH_3). On the other hand, it should be mentioned here that, using simulation software elaborated by Rockenbauer,[32] which permitted us to fit an experimental spectrum to a rapid exchange of the aminoxyls between the two phases, we were able to determine not only the EPR parameters of the methyl radical spin adducts in both water and micelles, but also the rate of the aminoxyl exchange between the two phases.

However, the SDS micelles represent a rather simplified biomembrane model, since the micelle core is strongly hydrophobic while intracellular media are aqueous. Thus, considering eventual *in vivo* applications of our traps, 4-PyOPN seems to be too hydrophilic and would remain in the extracellular environment, while the too lipophilic 4-ClPPN would be located in the phospholipid bilayer of cellular membranes. Nevertheless, a likely hypothesis is that PPN could partition first between the extracellular environment and the biomembrane, and then between the biomembrane and the intracellular medium. In this case, PPN could be a useful tool in the study of radical processes occurring in cells. Work is in progress in our laboratory to verify this hypothesis.

Experimental

The three spin trapping agents PPN, 4-PyOPN and 4-ClPPN were synthesised and purified in our laboratory as described previously.[24] SDS, iron(II) sulfate, sodium formate, hydrogen peroxide and EDTA (ethylenediaminetetraacetic acid) were purchased from Sigma Chemical Co. and used without further purification. Water and dimethyl sulfoxide (DMSO) were distilled twice before use.

The $CO_2^{\bullet -}$ and the $CH_3^{\bullet}$ radical spin adducts were obtained by carrying out a standard Fenton reaction in the presence of a nitrone and of sodium formate or of DMSO, respectively. To an aqueous solution of 0.05 mol dm^{-3} PPN or 4-PyOPN, or to a saturated solution of 4-ClPPN, containing EDTA (2 mmol dm^{-3}), $FeSO_4$ (5 mmol dm^{-3}), SDS (0–150 mmol dm^{-3}), and either sodium formate (0.2 mol dm^{-3}) or DMSO (10%), was added H_2O_2 (0.2%). The two carbon-centred radicals were then always generated in the bulk aqueous phase. In addition, $CO_2^{\bullet -}$ was unable to enter the micelles because of electrostatic repulsive forces with the sulfate groups of the micelle surface.

Samples were then transferred in capillary tubes and EPR spectra were recorded at 25 °C using a computer-controlled Bruker EMX spectrometer operating at X-band with 100 kHz modulation frequency, and equipped with an NMR gaussmeter for magnetic field calibration. The instrument settings were as follows: non-saturating microwave power, 10 mW; modulation amplitude, 0.08 mT; receiver gain ranging from 12×10^3 to 63×10^3; scan time, 180 s; time constant, 0.128 s.

The standard EPR signal simulations were achieved using the computer program elaborated by Dulling.[33] When the spin adducts were found to partition between the two phases, the EPR spectrum simulations were realised using the computer program elaborated by Rockenbauer,[32] which allows spectra resulting from two paramagnetic species in rapid equilibrium to be calculated.

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