An NMR relaxometric indicator of the formation of OH· radicals in Fenton-type reactions

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A gadolinium(III) paramagnetic complex bearing salicylate functionalities can be used to evaluate the OH· radical production by measuring water proton relaxation times.

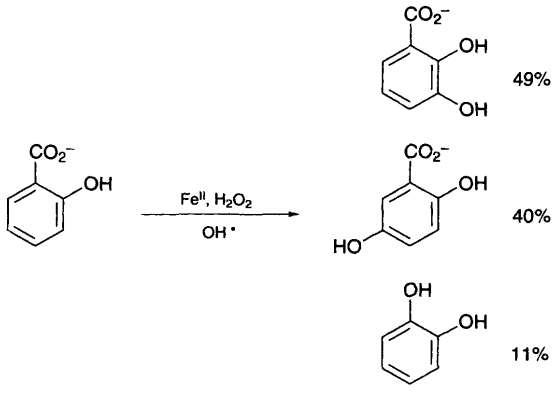
Recent years have witnessed a growing interest in the chemistry of OH· radicals the high reactivity of which is thought to be crucial to the onset of a number of relevant pathologies.[1] In biological systems, low molecular mass complexes of transition-metal ions are often supposed to act as catalysts for OH· radical production (Fenton-type reactions). On a laboratory scale, a Fenton-type reaction [eqn. (1)] may be set up by mixing an aqueous solution of an iron(II) edta complex and H_2O_2.[2]

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + OH^- + OH\cdot \qquad (1)$$

The occurrence of OH· radicals is commonly assessed *in vitro* by means of EPR spin-traps such as dmpo (5,5-dimethyl-1-pyrroline-*N*-oxide). In fact, the addition of an OH· radical to the C^2 carbon of this nitrone derivative yields 5,5-dimethyl-2-hydroxypyrrolidine-*N*-oxide.[3] This species may be quantified by measuring the intensity of its characteristic pseudo-quartet EPR signal arising from the coupling of the unpaired electron with ^{14}N (I = 1) and ^1H (I = 1/2) nuclei. Some years ago, an alternative HPLC procedure based on chemical transformation of salicylic acid upon OH· attack was reported.[4–6] As shown in Scheme 1, salicylic acid undergoes OH· attack to afford three dihydroxybenzene derivatives.

In this method, the yield of dihydroxybenzene derivatives provides an estimate of the extent of OH· radical formation in the system under study.

Here, we report on a novel NMR procedure based on the measurement of water proton longitudinal relaxation times T_1 in the presence of a paramagnetic gadolinium(III) complex containing, on its surface, two salicylate groups able to react with OH· radicals. Gadolinium(III) complexes are known to display a remarkable ability to enhance water proton relaxation rate and for this reason they have found a widespread application as contrast agents in magnetic resonance imaging.[7]



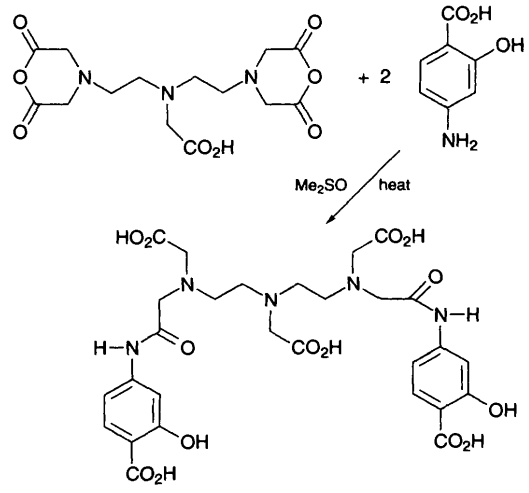
Scheme 1

The introduction of the salicylate functional groups on the surface of the gadolinium(III) complex was accomplished by the synthesis of a dtpa-derived ligand (L) *via* the reaction (Scheme 2) of dtpa bisanhydride with *p*-aminosalicylic acid.[8]

In the presence of a Fenton reagent, OH· attack on the salicylate substituents is expected to lead to their transformation into dihydroxybenzene moieties in an analogous manner to that shown in Scheme 1. Furthermore, the occurrence of Fe^{III} ions formed through eqn. (1) accelerates the oxidation of the catecholic moieties, which ultimately polymerise to melanin like structures. Melanins are insoluble materials, black or dark brown, derived from catechols, quinones, semiquinones and related derivatives linked through C–C and C–O–C bonds. The unpaired electrons in the semiquinone moieties display a very sharp EPR signal but do not lead to any paramagnetic relaxation of the water protons in which the insoluble melanin is suspended.[9,10]

As far as the formation of melanins from solutions of $Gd^{III}L$ is concerned, the polymerisation of the phenolic units results in the inclusion of the gadolinium(III) complex in the insoluble macromolecule. The removal of the paramagnetic $Gd^{III}L$ complex from the solution is easily assessed by measuring the solvent water proton relaxation times T_1.

Fig. 1 shows the decrease of water proton relaxation rates R_{1p} (= $1/T_{1p}$) for 2 mmol dm^{-3} solutions of $Gd^{III}L$ added to different amounts of $Fe^{II}(edta)^{2-}$ and H_2O_2. $R_{1p}(t)/R_{1p}(0)$ ratios are shown, where $R_{1p}(t)$ is the relaxation enhancement due to free $Gd^{III}L$ measured at time t and $R_{1p}(0)$ is the relaxation enhancement measured prior to addition of the Fenton reagent. R_{1p} values are obtained by subtracting the contribution to the relaxation enhancement due to the Fe^{III} ions formed during the reaction [eqn. (1)] and the diamagnetic contribution from the suspended melanin from the observed R_1 values. These contributions were estimated using the diamagnetic $La^{III}L$ complex in place of the paramagnetic $Gd^{III}L$ complex. From the data reported in Fig. 1, it is immediately evident that the



Scheme 2

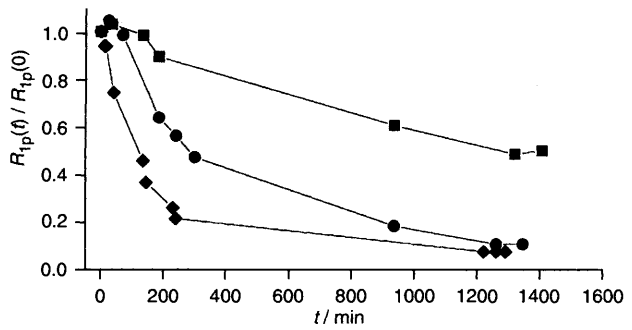


Fig. 1 Normalized water proton relaxation rates *vs.* time measured for 2 mmol dm^{-3} solutions of $Gd^{III}L$, with added Fenton reagents of different strength: (■) $[Fe^{II}(edta)^{2-}]$ = 0.5 mmol dm^{-3}, $[H_2O_2]$ = 23 mmol dm^{-3}; (●) $[Fe^{II}(edta)^{2-}]$ = 1.0 mmol dm^{-3}, $[H_2O_2]$ = 50 mmol dm^{-3}; (◆) $[Fe^{II}(edta)^{2-}]$ = 1.5 mmol dm^{-3}, $[H_2O_2]$ = 70 mmol dm^{-3}. The $R_{1p}(t)/R_{1p}(0)$ ratios were obtained by normalising R_{1p} values measured at time t with the R_{1p} values measured prior to addition of the Fenton reagent. T_1 values were measured at 298 K by using the 180–τ–90 pulse sequence on an instrument operating at 12 MHz. The reproducibility in T_1 measurements was ±1%. The reaction solutions were maintained at pH = 7.0 with 20 mmol dm^{-3} phosphate buffer.

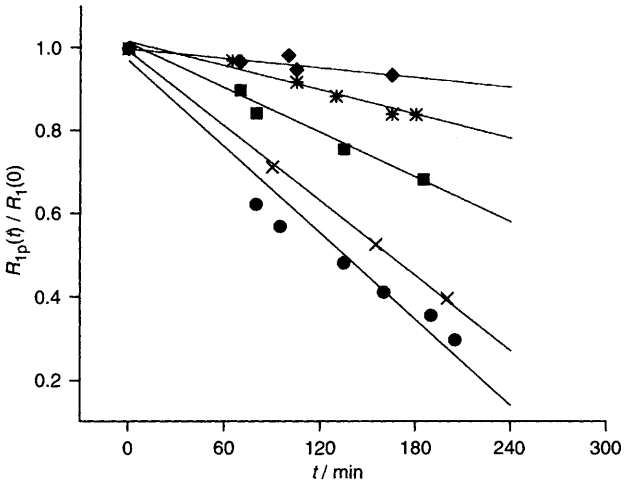


Fig. 2 $R_{1p}(t)/R_{1p}(0)$ *vs.* time of solutions made up with $[Gd^{III}L]$ = 2.0 mmol dm^{-3}, $[H_2O_2]$ = 50 mmol dm^{-3} and variable amounts of $Fe^{II}(edta)^{2-}$ [(●) 2.0, (×) 1.3, (■) 0.7, (*) 0.35, (◆) 0.07 mmol dm^{-3}]. Each data set has been fitted with a least-squares linear regression. The slopes of the straight lines obtained represent the $R_{1p}(t)/R_{1p}(0)$ decay constants k.

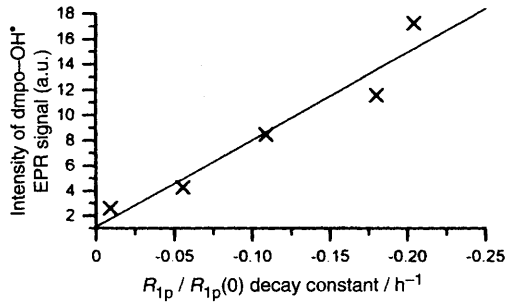


Fig. 3 EPR signal intensities of dmpo–OH· radical adduct *vs.* k [$R_{1p}(t)/R_{1p}(0)$ decay constant] obtained from solutions of $Gd^{III}L$ with added Fenton reagents at the strengths reported in Fig. 2

decrease with time of the $R_{1p}(t)/R_{1p}(0)$ ratios is related to the strength of the Fenton reagent.

A suitable method for the quantitative assay of the OH· radicals produced, and therefore of the strength of the Fenton reagent, is shown in Fig. 2. Here a plot of the $R_{1p}(t)/R_{1p}(0)$ ratio with time for five $Gd^{III}L$ solutions to which different amounts of $Fe^{II}(edta)^{2-}$ were added (concentration of hydrogen peroxide is constant) is shown. At 310 K, within the first 4 h of incubation, the decays of the normalised relaxation rates of the five solutions follow a fairly linear behaviour and the slopes of the straight lines obtained represent the $R_{1p}(t)/R_{1p}(0)$ decay constants (k). The k values obtained were found to correlate well with $Fe^{II}(edta)^{2-}$ concentrations. The strength of these five Fenton reagents has also been assessed by the dmpo spin-trap EPR method. As expected, the plot of the intensities of the EPR signal measured after 4 h *vs.* concentration of $Fe^{II}(edta)^{2-}$ clearly shows a linear relationship between the strength of the Fenton reagent and the extent of inclusion of OH· radicals into a dmpo molecule.

The k values related to the removal of $Gd^{III}L$ from the five solutions were plotted *vs.* the EPR signal intensities of the dmpo–OH· adduct at each Fenton reagent strength (Fig. 3). The straight line obtained clearly shows a direct proportionality between the NMR and EPR results.

The experiments were then repeated in the presence of 3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2*H*-1-benzopyran-2-carboxylic acid (trolox), a water-soluble chain-breaking antioxidant derived from vitamin E.[11] As expected, the rates of removal of $Gd^{III}L$ from solutions with added Fenton reagent of a given strength were found to decrease linearly with increasing trolox concentration.

In summary, whereas this assay essentially allows an easy route for the relative measurement of the OH· production between related experiments, we think that these observations may also provide a useful insight into the design of novel paramagnetic probes for MRI investigations, whose ability to enhance the water proton relaxation rate is dependent upon the formation of OH· radicals in their surroundings.

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References

1 S. Ylä-Herttuala, *Drugs Today*, 1994, **30**, 507; C. W. Olanow, *TINS*, 1993, **16**, 493; S. Bradamante, L. Barenghi, G. A. Giudici and C. Vergani, *Free Rad. Biol. Med.*, 1992, **12**, 193.
2 C. A. Rice-Evans, A. T. Diplock and M. C. R. Symons, *Techniques in Free Radical Research*, ed. R. H. Burdon and V. H. Van Knippenberg, Elsevier, Amsterdam, 1991.
3 G. R. Buettner, in *CRC Handbook of Methods for Oxygen Radical Research*, ed. R. A. Greenwald, CRC Press, Boca Raton, FL, 1985, pp. 151–155.
4 R. Richmond, B. Halliwell, J. Chauhan and A. Darbre, *Anal. Biochem.*, 1981, **118**, 328.
5 B. Halliwell, M. Grootveld and J. M. C. Gutteridge, *Method Biochem. Anal.*, 1988, **33**, 89.
6 M. Grootveld and B. Halliwell, *Biochem. Pharmacol.*, 1988, **37**, 271.
7 R. B. Lauffer, *Chem. Rev.*, 1987, **87**, 901.
8 S. Aime, M. Botta, M. Fasano and E. Terreno, *Spectrochim. Acta, Part A*, 1993, **49**, 1315.
9 G. Prota, *Melanins and Melanogenesis*, Academic Press, San Diego, 1992.
10 S. Aime, M. Fasano, E. Terreno, C. Sarzanini and E. Mentasti, *Magn. Reson. Imag.*, 1991, **9**, 963.
11 G. Cao, H. M. Alessio and R. G. Cutler, *Free Rad. Biol. Med.*, 1993, **14**, 303.

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