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Effectiveness and Toxicity of Several DTPA Broadening Agents for Biological ESR Spectroscopy

A. N. ZAPLATIN,*,† KENT A. BAKER,*,† AND F. W. KLEINHANS*,‡

*Department of Physics, IUPUI, Indianapolis, Indiana 46202; and †Cryobiology Research Institute, Methodist Hospital of Indiana, Indianapolis, Indiana 46202

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The effectiveness of a standard ESR broadening agent, potassium trioxalatochromiate (CrOx), for use with the spin-label tempone, was compared to that of diethylenetriaminepentaacetic acid (DTPA) containing an ion (Gd, Cr, Mn, Fe) with a large magnetic moment. Signal attenuation, line broadening, toxicity, and cell membrane permeability were compared. As a broadening agent, CrOx was most effective, followed by Fe–DTPA. CrOx proved mildly toxic while Gd-DTPA and Fe–DTPA were virtually nontoxic. The human red blood cell membrane was tested for permeability to Fe– and Gd–DTPA and found to be impermeable to both. In situations where toxicity to cells is critical, the DTPA chelates, particularly Fe–DTPA, may prove an acceptable substitute for CrOx. © 1996 Academic Press, Inc.

INTRODUCTION

ESR spectroscopy of cells has been widely used to study a variety of biophysical problems. Lipophilic and protein labels have been used to directly probe cell membranes and aqueous labels have been used to probe the intracellular environment and membrane permeability. In this latter technique, an aqueous label, which permeates the membrane, is used to label all aqueous components of a cell suspension. Then, a membrane impermeant magnetic broadening agent is used to broaden the extracellular label signal to near extinction, thus allowing measurement of intracellular aqueous compartments (*1*). This technique has proven useful for the investigation of the microviscosity of the cell cytoplasm (*1–4*), for cellular volume measurements (*3, 5–7*) and for membrane permeability measurements (*8–10*).

The choice of a broadening agent is an important part of these experiments. The ideal broadening agent is one which yields significant signal suppression, is impermeant to cell membranes, and is nontoxic to cells. Early ESR experiments (*1, 2, 4*) used $NiCl_2$ and $K_3Fe(CN)_6$, which in many cases are damaging to cells. In 1979, Berg and Nesbitt (*11*) and Yager *et al.* (*12*) introduced a superior broadening agent, potassium trioxalatochromiate (CrOx). It was found to be twice as effective as $K_3Fe(CN)_6$. Although the toxicity of CrOx is much less than that of $NiCl_2$ and $K_3Fe(CN)_6$, it is not ideal. For instance, it can quickly immobilize up to 25% of human sperm (*7*).

A number of other broadening agents have been suggested or found useful in biological ESR studies. Sodium–manganese–EDTA has been used to investigate osmoregulation in cyanobacteria (*13*), MnO–EDTA has been suggested for the measurement of mammalian sperm intracellular water (*14*), and KMn–EDTA has been used in an investigation of the permeability of human red blood cells (RBC) (*10*). Recently, $Cr(maltolate)_3$ was proposed when a neutral broadening agent is desired (*15*). This latter agent may also prove useful in situations where a low solution osmolality is important, a consideration which is discussed in more detail below.

It is important for many cell experiments utilizing a broadening agent to be able to control the osmolality of the cell environment. The osmolality of the environment can be changed by adjusting concentrations of the different components of the cell medium; however, the more the broadening agent contributes to the osmolality, the less flexibility there is to control the osmolality and other parameters of the solution. The broadening agent, for example, determines the lower limit of the osmolality attainable in Boyle–van't Hoff experiments in which the cell volume is measured as function of osmolality (*16*). Thus, other things being equal, the lower the osmolality of the broadening agent the better.

The modest toxicity and high osmolality of the commonly used CrOx were the motivation for trying to find a nontoxic, yet effective broadening agent in a class of DTPA compounds that contain ions with a large magnetic moment. Chemicals of this class are extensively used in NMR imaging (*17*); e.g., Gd–DTPA is widely used for the diagnosis of cerebral tumors (*18*) and multiple sclerosis (*19*).

This study reports an investigation and comparison of the broadening properties, membrane permeability, and toxicity of Fe(III) disodium salt dihydrate DTPA (Fe–DTPA), Cr(III) disodium salt hexahydrate DTPA (Cr–DTPA),

‡ To whom correspondence should be addressed.

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Mn(II) trihydrogen salt monohydrate DTPA (Mn–DTPA), and Gd(III) dihydrogen salt hydrate DTPA (Gd–DTPA) to those of CrOx. Membrane permeability was studied using human RBC because they are readily available. Human sperm were chosen for the toxicity study because of their importance to our cryobiology research. Additionally, computer-assisted semen analysis (CASA) (*20*) is a very convenient and sensitive method of assessing toxicity.

MATERIALS AND METHODS

ESR Measurements

Electron spin resonance measurements were conducted on a Varian X Band E109 spectrometer with a dual rectangular cavity. Data acquisition and handling were performed with an HP 330 data system with custom software for biological spin-label applications. The aqueous probe tempone (Molecular Probes, Eugene, Oregon) at a concentration of 2 m*M* was used for all experimental measurements. Although some signal broadening occurs at this concentration due to label–label interactions, this drawback is compensated for by an increase in the sensitivity that a 2 m*M* concentration yields (*7, 9*). Tempone (4-Oxo-TEMPO) is a common nitroxide free radical that yields a triplet spectrum due to the ^{14}N hyperfine splitting.

The spectrometer settings were power, 20 mW (10 mW per cavity); magnetic field, 3269 G; modulation amplitude, 0.5 G; amplifier time constant, 0.016 s; sweep width, 40 G; and sweep time, 200 s. All samples were placed in Clay Adams (Parsipany, New Jersey) 50 μL disposable glass micropipettes and sealed with Critoseal (Monojet Scientific, St. Louis, Missouri). The same settings were also used for the cellular measurements. The intracellular signals were obtained by digital subtraction of the signals obtained from cell and cell-free samples, respectively (*7*).

Broadening Effect Study

CrOx was prepared using the method of Bailar and Jones (*21*). Fe–, Cr–, Mn–, and Gd–DTPA were prepared by dilution of the appropriate salts (Aldrich Chemical Company Inc., Milwaukee, Wisconsin) in deionized water. The pH of the broadening solutions ranged from 1.8 for 70 m*M* Gd–DTPA to 7.3 for 80 m*M* Cr–DTPA. The middle-field peak-to-peak amplitude (h) and peak-to-peak linewidth (W) were determined using a quadratic, least-squares fit to the tips of the positive- and negative-going first-derivative ESR peaks. These were measured as a function of the broadening-agent concentration. No correction was made for line overlap, which may introduce small errors at the largest line broadenings.

To characterize the efficiency of the broadening agent, we define attenuation (A) as $A = h(0)/h(C)$ where h is the peak-to-peak amplitude of the ESR signal and C is the concentration (millimolarity) of the broadening agent.

RBC and Sperm Samples

Sperm samples were obtained by masturbation from normal, human research donors after at least two days of sexual abstinence. Motile sperm were then separated using a discontinuous Percol gradient and rinsed twice in TL-Hepes buffer (290 mOsm). Human RBC samples were obtained by venepuncture from healthy donors into 10 cc vacutainer tubes with acid citrate dextrose anticoagulant. The cells were then rinsed twice in phosphate-buffered saline (PBS, 290 mOsm) prior to use.

RBC Membrane Permeability Measurements

The membrane permeability of the broadening agent was determined by observing the middle-field, intracellular tempone EPR signal at 10 min intervals over a period of one hour. If a membrane is permeable to the broadening agent, a decrease of the intracellular signal amplitude will occur over time as the broadening agent leaks into the cell. (Reduction of the spin label would, of course, also lead to a decrease in intracellular signal amplitude.) To perform this experiment, RBC were resuspended in a TL-Hepes buffer with the broadening agent, tempone, and NaOH (to bring the pH to 7.4).

Toxicity Study

Sperm motility was utilized as a criterion to estimate the toxicity of the broadening agents. Toxicity measurements for 50 m*M* CrOx were previously done by Kleinhans *et al.* (*7*). Toxicity measurements of 100 m*M* Gd–DTPA and 85 m*M* Fe–DTPA to human spermatozoa were conducted at a temperature of 37°C over a period of 2.5 h. Sperm were suspended in TL-Hepes and broadening agent (adjusted to pH 7.4 with NaOH prior to cell addition). Sperm motility of test and control samples was measured initially and at 30 min intervals using the CellSoft computer-assisted semen analysis system (Cryo Resources, Ltd., New York) (*20*). Results are presented as the ratio of test to control motility at each time.

pH Effect and Osmolality Measurements

The pH of each of the most concentrated metal–DTPA (in water plus tempone) broadening solutions was measured and the most acidic (Gd–DTPA) was further tested for the effect of pH on broadening as follows. Seventy millimolar Gd–DTPA plus 2 m*M* tempone were prepared in pure water and in TL-Hepes buffer. In water, the 70 m*M* Gd–DTPA (plus tempone) yielded a pH of 1.8, and, in the TL-Hepes solution, the pH was adjusted to pH 7.4 using NaOH. The

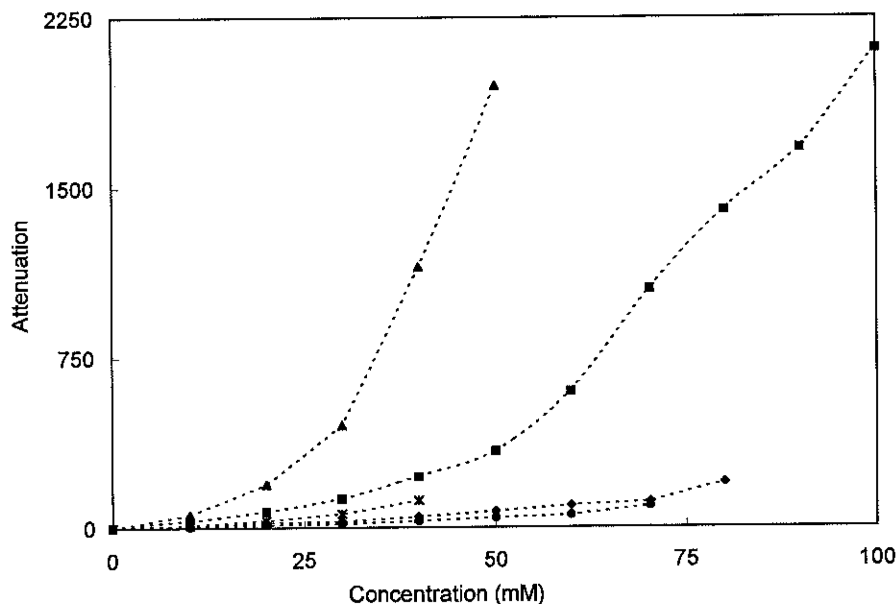

FIG. 1. Attenuation of 2 mM tempone ESR signal strength versus broadening-agent molarity (▲, CrOx; ■, Fe–DTPA; ★, Mn–DTPA; ♦, Cr–DTPA, ●, Gd–DTPA). Attenuation is defined as $A = h(0)/h(C)$, where h is the peak-to-peak amplitude of the ESR signal and C is the molarity of the broadening agent. Attenuation was used as a measure of broadening ability.

broadening properties of these two test solutions were then compared.

All sample osmolalities were measured with a freezing point depression osmometer (3D2 Advanced Instrument, Inc. Norwood, Massachusetts) with an accuracy of ±5 mOsm.

Line Broadening

Eaton and Eaton have published a comprehensive review of spin-label interactions with transition metals (*22*). Here we consider only a few points. We assume that the spin label and broadening agent are present in dilute concentration, and, thus, the interactions between the spin label and broadening agent are pairwise. Typically, for systems in liquid solution, as is the case here, the lines are largely Lorentzian in nature (*23*). Then, in a wide variety of cases using nitroxide labels and transition metal broadening agents, it is found that the peak-to-peak (PTP) linewidth (W) is proportional to the concentration (C) of the broadening agent (*22*)

$$W = \alpha C + \beta, \qquad [1]$$

where $W(0) = \beta$ is just the unbroadened linewidth of the tempone line. The maximum value of absorption is inversely proportional to W and the peak-to-peak amplitude (h) of the first-derivative signal is, in turn, inversely proportional to the square of the linewidth (*23*)

$$h = kW^{-2} = k(\alpha^2C^2 + 2\alpha\beta C + \beta^2)^{-1}, \qquad [2]$$

where k is simply the constant of proportionality. The attenuation (A) is given by

$$A = h(0)/h(C) = W^2(C)/W^2(0) = \alpha^2\beta^{-2}C^2 + 2\alpha\beta^{-1}C + 1, \qquad [3]$$

which can be expressed as

$$A = aC^2 + bC + 1. \qquad [4]$$

Thus, attenuation vs molarity is described by a second-degree polynomial, but with the constraint that a and b are related and given by the expressions $a = \alpha^2\beta^{-2}$ and $b = 2\alpha\beta^{-1}$ in which α is the only independent fitting parameter. In practice, nitroxyl lines in solution are not purely Lorentzian and the proportion of Gaussian-to-Lorentzian contribution changes as the line is broadened. Thus, the relationship between attenuation and molarity may not be strictly second order.

RESULTS

Broadening Effect Study

The results of the broadening effect studies are presented in Fig. 1 and Fig. 2. Figure 1 shows the dependence of the tempone ESR signal attenuation on the concentration

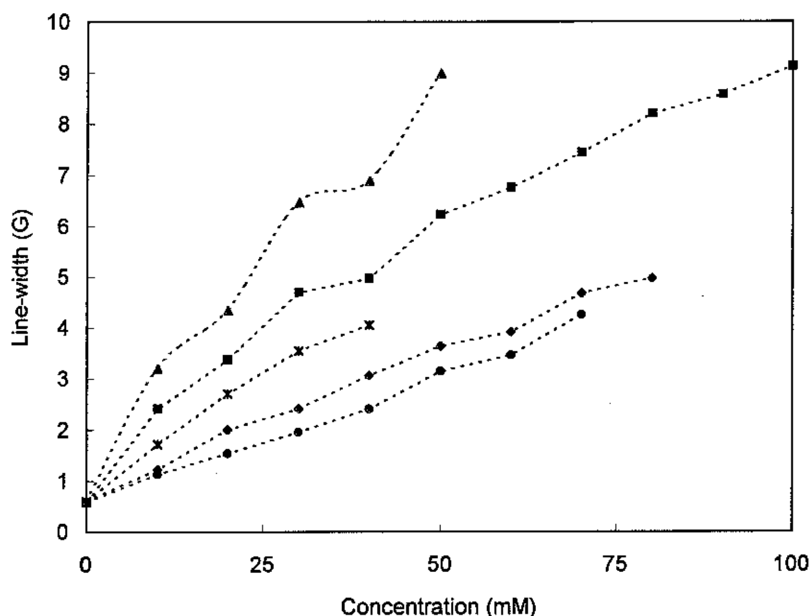

FIG. 2. Peak-to-peak ESR linewidth (gauss) of 2 mM tempone as function of broadening-agent molarity (▲, CrOx; ■, Fe–DTPA; ★, Mn–DTPA; ♦, Cr–DTPA; ●, Gd–DTPA).

(molarity) of the broadening agent, and Fig. 2 shows the linewidth of the signal as a function of the concentration of the broadening agent. Both graphs show that CrOx is the most effective broadening agent. At a concentration of 50 mM, CrOx attenuates the tempone signal 1950 times. From most to least effective, the DTPA compounds are ranked in the order Fe–, Mn–, Cr–, and Gd–DTPA, respectively.

The linewidth was observed to be a linear function of the broadening-agent concentration. A typical linear fit ($W = 8.11 \times 10^{-2}$ G mM^{-1} C + 1.62 G, $r^2 = 0.968$) to the experimental data for Fe–DTPA is shown in Fig. 3. In all cases, the coefficient of determination (r^2) was greater than 0.96. The unbroadened linewidth, $W(0) = \beta$, of the midfield tempone triplet was 0.59 G.

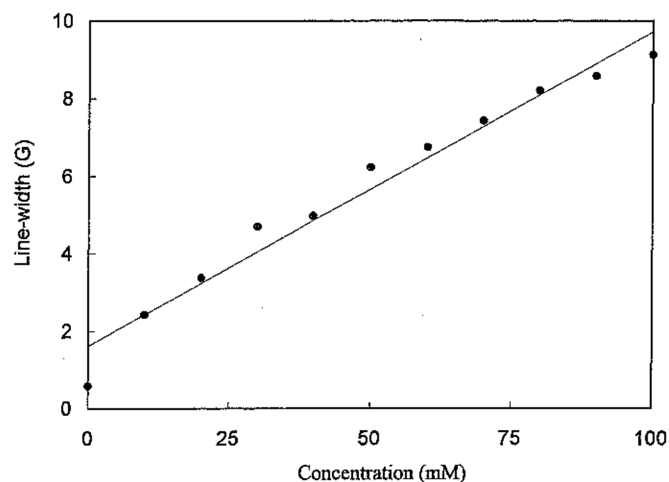

FIG. 3. Peak-to-peak ESR linewidth of the midfield, 2 mM tempone signal as a function of Fe–DTPA molarity. The solid curve shows a linear fit to the experimental data: $W = 8.11 \times 10^{-2}$ G mM^{-1} C + 1.62 G.

Attenuation was found to obey the relationship

$$\text{Attenuation} = \frac{h(0)}{h(C)} = aC^2 + bC + 1, \qquad [5]$$

where a and b are constrained as described above. The results of the quadratic fit for all broadening agents are summarized in Table 1. Figure 4 illustrates the agreement between a quadratic function and the experimental data for Fe–DTPA.

TABLE 1

ESR Signal Attenuation (A) as a Function of Concentration (Millimolarity, C) of the Broadening Agent $A = h(0)/h(C) = aC^2 + bC + 1$

Agent	α	r^2	a	b
CrOx	0.503	0.987[a]	0.7352	1.715
Fe–DTPA	0.259	0.991	0.1952	0.884
Mn–DTPA	0.142	0.997	0.0585	0.484
Cr–DTPA	0.089	0.982	0.0231	0.304
Gd–DTPA	0.068	0.987	0.0135	0.232

Note. The quadratic term dominates, and, thus, a is a measure of the relative effectiveness of each broadening agent. The parameters a and b are related to the fitting parameter (α) by $a = \alpha^2\beta^{-2}$ and $b = 2\alpha\beta^{-1}$, where $\beta = W(0) = 0.5862$ G.

[a] Coefficient of determination, r^2.

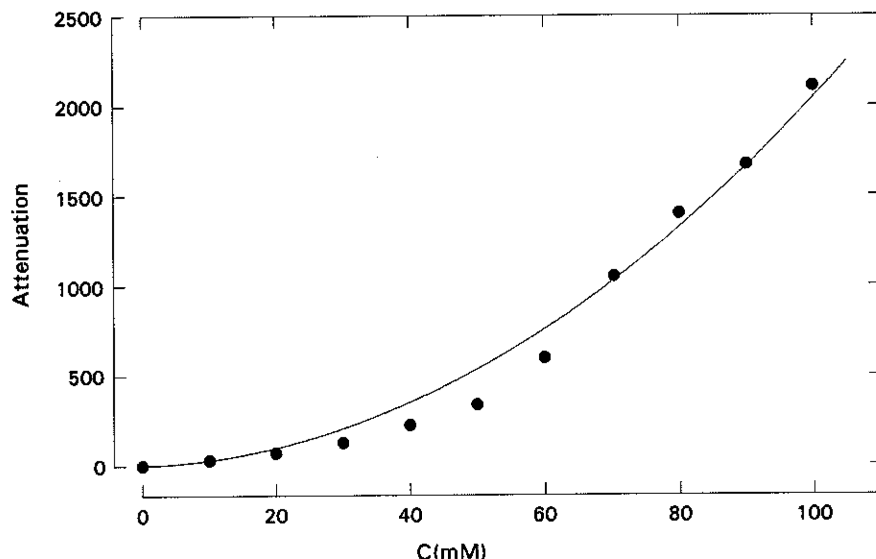

FIG. 4. ESR signal attenuation, $h(0)/h(C)$, versus Fe–DTPA concentration. The solid curve shows a quadratic fit to the data.

Membrane Permeability Experiment

In the human RBC membrane permeability experiments, the intracellular, midfield signal amplitude varied by less than 4% during the course of a one hour exposure of the cells to Fe–DTPA or Gd–DTPA. Thus, Fe–DTPA and Gd–DTPA do not cross the RBC membrane.

Toxicity Study

The toxicity study showed that 85 mM Fe–DTPA and 100 mM Gd–DTPA were virtually nontoxic, resulting in a statistically insignificant loss of cell motility after one hour. Experimental data are presented in Fig. 5.

pH and Osmolality Study

pH does not significantly affect the performance of the DTPA compounds as broadening agents. Seventy millimolar Gd–DTPA at pH 1.8 and 7.4 gave identical signal attenuation to within 5% which is less than the expected uncertainties due to sample and spectrometer errors. The midfield peak-to-peak linewidth (4.1 G) differed by only $3\frac{1}{2}$% be-

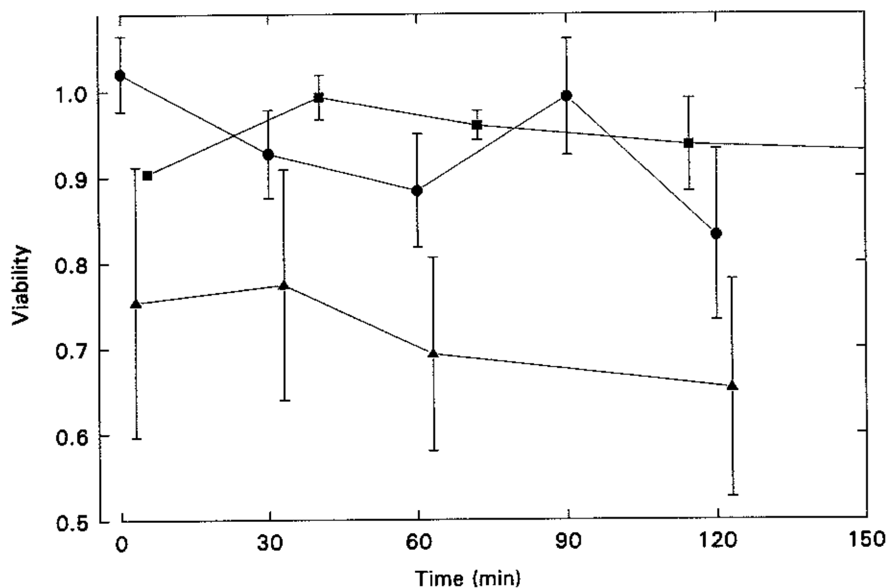

FIG. 5. Toxicity data: The vertical axis represents the ratio of the motile cells in the experimental sample, containing the broadening agent (▲, 50 mM CrOx; ■, 85 mM Fe–DTPA; ●, 100 mM Gd–DTPA), to that of the control sample without broadening agent. The CrOx data are from Kleinhans *et al.* (7). (Means ± SEM)

TABLE 2
Osmolality and pH of Broadening Agents

Agent	Osmolality (mOsm) (@ 40 m*M*)	pH
Cr–DTPA	160	7.3 @ 80 m*M*
CrOx	115	6.0 @ 50 m*M*
Fe–DTPA	104	5.1 @ 100 m*M*
Gd–DTPA	52	1.8 @ 80 m*M*
Mn–DTPA	50	2.6 @ 40 m*M*

tween the two samples. A less rigorous examination of Fe–DTPA and CrOx broadening yielded similar, insignificant pH differences.

The pH and osmotic coefficient of the different broadening solutions are shown in Table 2. As expected from the chemical formula, the sodium salt DTPAs (Cr and Fe) had higher osmolalities than the hydrogen salt DTPAs (Mn and Gd).

DISCUSSION

The results of this work show that CrOx is the most effective broadening agent among those tested. For a given degree of attenuation, the DTPA chelates offer no advantage of lower osmolality over CrOx. The linear proportionality of linewidth and quadratic proportionally of attenuation to transition metal concentration, respectively, are consistent with previous observations in similar systems (*22*). Comparison of the toxicity of Gd–DTPA and Fe–DTPA to human sperm with that of CrOx, reported by Kleinhans *et al.* (*7*), shows that DTPA compounds are superior to CrOx in terms of lower toxicity.

In the range tested, pH did not affect broadening, probably because tempone is a neutral molecule unaffected by ionic attraction or repulsion. Yager *et al.* (*12*) discuss pH effects in some detail for the tempamine–CrOx system and also report that buffer concentration affects broadening. This latter effect was not investigated in this study.

Based on our results, we believe that the DTPA chelates, particularly Fe–DTPA, may be useful as a broadening agent with tempone for ESR biological studies where toxicity is a primary concern. However, because of their reduced broadening effectiveness compared with CrOx, relatively high cell cytocrits (>10%) are required to yield good separation of the intra- and extracellular signals.

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