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Electron spin resonance spectroscopy studies on reduction process of nitroxyl radicals used in molecular imaging

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Abstract: Electron spin resonance (ESR) spectroscopy studies on the reduction process of nitroxyl radicals were carried out for 1mM concentration of ^{14}N-labeled nitroxyl radicals in 1 mM concentration of ascorbic acid as a function of time. The half life time and decay rate were estimated for 1mM concentration of ^{14}N labeled nitroxyl radicals in 1 mM concentration of ascorbic acid. From the results, the increase in half life time and decrease in decay rate were calculated for TEMPONE compared with TEMPO and TEMPOL radicals, which indicates the higher stability of TEMPONE radical. The observed radical scavenging activity is also higher for TEMPONE radical. The ESR spectrum was also recorded for 1mM concentration of ^{14}N-labeled nitroxyl radicals in pure water and the ESR parameters, line width, hyperfine coupling constant, g-factor, signal intensity ratio and rotational correlation time were obtained. These results indicate that the TEMPONE radical has narrowest line width and fast tumbling motion compared with TEMPO and TEMPOL. Therefore, this study reveals that the TEMPONE radical can act as a good redox sensitive spin probe for molecular imaging.

Keywords: Electron Spin Resonance Spectroscopy, Nitroxyl Radicals, Ascorbic Acid, Half Life Time, Decay Rate

1. Introduction

Stable piperidine and pyrrolidine nitroxyl radicals have been widely used in biophysical studies as probes for measuring redox metabolism. It can serve as redox probes providing information regarding cellular redox state, when they are introduced into the sample and the measurement of their spectra can be used to detect the structural changes, motional characteristic of the environment in the sample [1,2]. Nitroxyl radicals have also been used as a spin probe for low frequency in vivo electron spin resonance (ESR) experiments to estimate the biological redox status in living experimental animals. The biomedical applications of redox imaging provide the quantification of the nitroxyl radical concentration in tissues, in vivo redox reactions, reactive oxygen species and intracellular antioxidants [3,4].

ESR is a very suitable and versatile method to provide information on the microenvironment of the spin probe, the recorded ESR parameters are dramatically affected by the physical properties such as polarity, viscosity and dynamics of the surrounding space of the probe [5]. The ESR imaging technique can detect free radicals both in vitro and non-invasively in vivo with high sensitivity, which is used to investigate the free radical distribution and metabolism in tissues, organs and the whole body of small animals [6,7]. The ESR X-band frequency (~9GHz), the magnetic interactions of nitroxide spin labels are extremely sensitive to motion on the nanosecond time scale, which is relevant to the dynamics of bio-molecules [8]. The spin labels, especially nitroxyl radicals exhibit an ESR spectrum which is very sensitive to slight changes in the environment and therefore have been used to characterize the interaction of labeled molecules [9]. In ESR measurements, organ reducing activity was monitored by injecting a stable paramagnetic spin probe into the animals. This spin probe was converted into the corresponding hydroxylamine by one electron reduction and loses its ESR signal in organs. The measurement of ESR signal intensity is equal to the measurement of organ reducing activity against radicals, which is a major factor of organ antioxidative activity. Therefore, the measurement of reducing or oxidizing

activity by ESR technique using nitroxyl spin probes may provide various information about the reductive or oxidative environments in living tissues, free radical metabolism, in vivo redox status and biological status [10-22].

Reduction of the paramagnetic 3-carbamoyl PROXYL by ascorbic acid to the diamagnetic by hydroxylamine was monitored from a sequence of temporal images, acquired using the three imaging modalities ESR imaging, Overhauser-enhanced magnetic resonance (OMR) imaging, Magnetic resonance (MR) imaging [23]. Utsumi et al. reported that the Dynamic nuclear polarization (DNP) properties of nitroxyl radicals used in Overhauser-enhanced magnetic resonance imaging for simultaneous molecular imaging of redox reactions [24]. Recently, the stable nitroxyl radicals have been used as redox sensitive probe for in vivo ESR and OMR imaging techniques [25-31]. This proposed work determines the half life time and the signal decay rate for the piperidine nitroxyl radicals. Moreover, in order to understand the reduction process and find the suitable nitroxyl spin probe among the piperidine nitroxyl radicals for in vivo/in vitro ESR and OMR imaging techniques. Here, we report the ESR spectroscopy studies on the reduction process of 1mM concentration of ^{14}N-labeled TEMPONE, TEMPO and TEMPOL in 1 mM concentration of ascorbic acid.

2. Materials and Methods

2.1. Chemicals

The spin probes, 4-oxo-2,2,6,6- tetramethyl –piperidine -1-oxyl (TEMPONE), 2,2,6,6-tetramethyl piperidine -1-oxyl (TEMPO), 4-hydroxy -2,2,6,6-tetramethyl-piperidine-1-oxy (TEMPOL) and ascorbic acid were purchased from Sigma Aldrich Chemical Co, St. Louis, MO, USA.

2.2. ESR Measurements

ESR spectra were recorded for 1 mM concentration of ^{14}N labeled TEMPONE, TEMPO and TEMPOL in 1 mM concentration of ascorbic acid as a function of time using a Bruker EMS plus X-band ESR spectrometer by varying the magnetic field, 342-352 mT; with modulation frequency, 100 kHz; field modulation amplitude, 0.2 mT; conversion time, 10 ms; radio-frequency power, 2 mW; receiver gain, 1000; sweep width, 10 mT; sweep time, 10 s; point field resolution, 1024; and microwave frequency, 9.86 GHz. The ESR spectra were recorded in the first derivative mode at 27ºC. The temperature was controlled using a controller with water as a coolant. The ESR spectrum was also recorded for 1 mM concentration of ^{14}N labeled nitroxyl radicals in pure water with an accuracy of ~±0.5 μT.

3. Results and Discussion

The ring structure and abbreviations of ^{14}N-labeled TEMPONE, TEMPO and TEMPOL are given in Table 1. The ESR parameters, such as the line width, g-factor, hyperfine coupling constant and rotational correlation time for 1 mM concentration of ^{14}N-labeled TEMPONE, TEMPO and TEMPOL in pure water are listed in Table 2. The paramagnetic nitroxyl radical was converted into the paramagnetic hydroxylamine form in the reaction with ascorbic acid, which leads to the reduction of ESR signal intensity. Fig 1 shows the schematic diagram of the reduction process of nitroxyl radical. The ESR spectra of 1 mM concentration of ^{14}N-labeled TEMPONE, TEMPO and TEMPOL in pure water and 1mM concentration of ascorbic acid as a function of time (t) are shown in Figs 2-4.

Table 1. *The ring structure and abbreviations of nitroxyl radicals.*

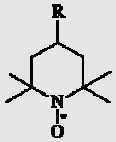
Ring structure	Substituents (R)	Abbreviations
	=O	TEMPONE
	-H	TEMPO
	-OH	TEMPOL

Table 2. *ESR parameters of 1mM ^{14}N labeled TEMPONE, TEMPO and TEMPOL in pure water.*

Sample	Line width ΔB (μT)	Hyperfine coupling constant (mT)	g-factor	Signal intensity ratio (h_0/h_{-1})	Rotational correlation time τ_R (s) ($\times 10^{-11}$)
TEMPONE	136	1.725	2.0068	1.041	1.817
TEMPO	151	1.593	2.0070	1.044	2.167
TEMPOL	185	1.686	2.0078	1.075	4.444

3.1. Line Width

The observed line width values are given in Table 2. The line width broadening is due to the dipolar and spin exchange interactions of agent concentrations. These results agree well with the previous studies [32-35]. The TEMPONE has a narrow line width compared with TEMPO and TEMPOL radicals. The obtained line width values were ~11 and 36% higher for TEMPO and TEMPOL compared with TEMPONE radical. The narrowest line width of TEMPONE radical indicates that the interaction between the carbonyl group of TEMPONE and water proton is less, but the interaction between the OH group of TEMPOL and water proton is more [36]. The line width value of TEMPO shows only 11% increase, which is due to the interaction between the substituent (R), hydrogen atom of TEMPO and water proton.

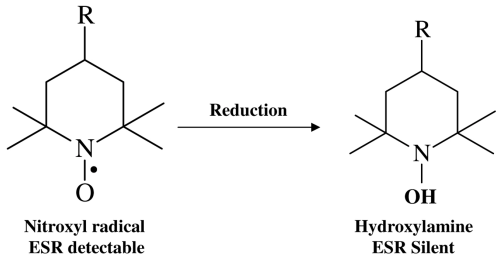


Figure 1. *Schematic diagram of reduction process of nitroxyl radical, R represents substituents.*

3.2. Hyperfine Coupling Constant and g-factor

Table 2 shows the hyperfine coupling constant and g-factor for 1 mM concentration of ^{14}N-labeled TEMPONE, TEMPO and TEMPOL radicals. The hyperfine coupling constants for the nitroxyl radicals agree well with the previous study [33,36]. The obtained hyperfine coupling constant values indicate that the Fermi contact interaction is less for TEMPO compared with TEMPONE and TEMPOL. The g-factor value indicates that the isotropic nature of the system.

3.3. Signal Intensity Ratio

The signal intensity ratio was calculated from the height of the central line and high field line of the ESR spectrum. The signal intensity ratio values of 1mM concentration of ^{14}N-labeled TEMPONE, TEMPO and TEMPOL in pure water are listed in Table 2. The ESR signal intensity ratio value becomes unity for all nitroxyl radicals in pure water, which reveals that the homogenous nature of the samples. These results agree well with the reported values [36].

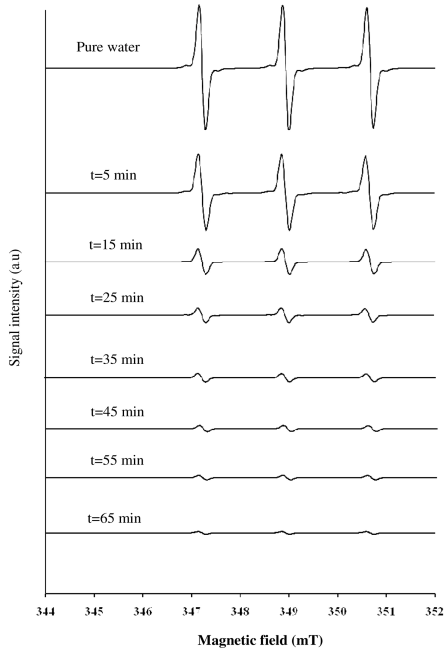


Figure 2. *ESR spectra of 1mM concentration of ^{14}N-labeled TEMPONE in pure water and 1mM concentration of ascorbic acid as a function of time (t).*

3.4. Rotational Correlation Time

Conventional ESR spectroscopy can detect changes in the rotational correlation time (τ_R) of spin probes ranging from 10^{-12} to 10^{-9} s, which corresponds to the lifetime of the probe in a given orientation. In this motional range, the ESR spectrum of nitroxyl radical consists of three lines, and τ_R can be calculated according to the method of Knowles et al. [36,37].

$$\tau_R = 6.5\times10^{-10}\ \Delta B_0[(h_0/h_{-1})^{1/2} - 1]$$

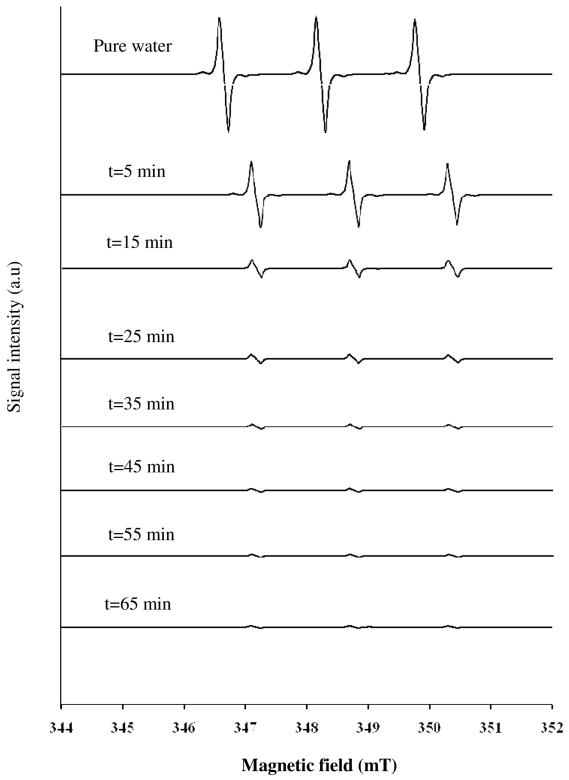


Figure 3. *ESR spectra of 1mM concentration of ^{14}N-labeled TEMPO in pure water and 1mM concentration of ascorbic acid as a function of time (t).*

Where, h_0 and h_{-1} are the heights of the central and high field line in the ESR spectrum, respectively, and ΔB_0 is the line width of the central line in Gauss. The rotational motion of the spin probe was assumed to be isotropic.

The rotational correlation time for 1mM concentration of ^{14}N-labeled TEMPONE, TEMPO and TEMPOL in pure water is shown in Table 2. The rotational correlation time agrees well with the previous study [36]. The decrease in rotational correlation time was observed for TEMPONE compared with TEMPO and TEMPOL in pure water. These results show that the TEMPONE radical has fast tumbling motion compared with TEMPO and TEMPOL radical. The fast tumbling motion confirms the less interaction between the carbonyl group of TEMPONE radical and water protons. The narrowest line width also indicates the fast tumbling motion of the TEMPONE radical.

3.5. Reduction Process and Stability

Nitroxyl radicals react with reductants and their reduction process depend on the basic structure, chemical and physical properties of the nitroxyl radicals. Figs 2-4 show the ESR spectrum of 1 mM concentration of ^{14}N-labeled TEMPONE, TEMPO and TEMPOL in 1 mM concentration of ascorbic acid as a function of time, which demonstrate the reduction process of nitroxyl radicals towards the ascorbic acid. During the reduction process, the nitroxyl radical was converted into hydroxylamine and loses its paramagnetic nature, which leads to the ESR signal decay.

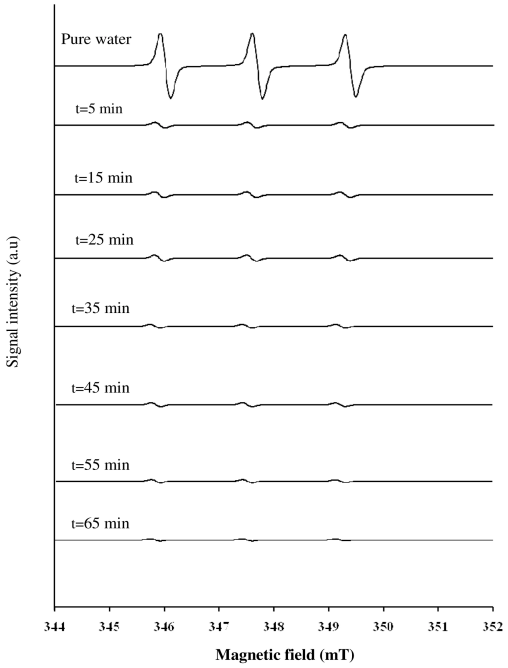


Figure 4. *ESR spectra of 1mM concentration of ^{14}N-labeled TEMPOL in pure water and 1mM concentration of ascorbic acid as a function of time (t).*

3.6. Half Life Time and the Decay Rate

The ESR signal intensity values were fitted with a simple model of an exponential decay equation and an added constant offset, which is used to determine the half life time ($t_{1/2}$) and the decay rate ($1/\tau$) of the nitroxyl radical in ascorbic acid [10, 40]. The ESR spectral intensity I (t) can be expressed as

$$I(t) = I_0 e^{-t/\tau} + N$$

where, I_0 is the initial value of ESR signal intensity, t is the time and $1/\tau$ is the decay rate.

Fig 5a shows the fitted exponential decay curve for 1mM concentration of ^{14}N-labeled TEMPONE, TEMPO and TEMPOL in 1mM concentration of ascorbic acid at various time intervals. The curve fitting shows the best correlation (R^2>0.99). The half life time of 1mM concentration of ^{14}N-labeled TEMPONE, TEMPO and TEMPOL in 1mM concentration of ascorbic acid as a function of time was calculated by the equation

$$t_{1/2} = \tau \ln(2)$$

The half life time and decay rate values for TEMPONE, TEMPO and TEMPOL are listed in Table 3. The values given are the averages of three repeated experiments. The increase in half life time and decrease in decay rate were obtained for TEMPONE compared with TEMPO and TEMPOL radical, which indicates the higher stability of TEMPONE radical.

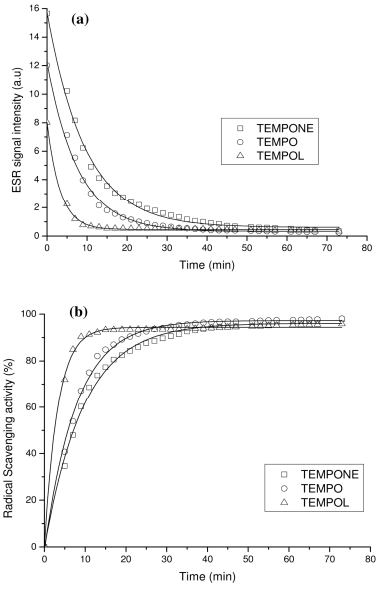


Figure 5a. *The exponential decay curve fit for the ESR signal intensity and* ***b*** *the radical scavenging activity of 1mM concentration of ^{14}N-labeled TEMPONE, TEMPO, TEMPOL in pure water and 1mM concentration of ascorbic acid at various time intervals.*

Table 3. *The half life time and decay rate of 1mM ^{14}N labeled TEMPONE, TEMPO and TEMPOL in 1 mM concentration of ascorbic acid.*

Sample	Half life time $t_{1/2}$ (min)	Signal decay rate $1/\tau$ (min^{-1})
TEMPONE	6.662	0.104
TEMPO	5.412	0.128
TEMPOL	2.263	0.306

3.7. Radical Scavenging Activity

The Radical scavenging activity (RSA) was expressed as the inhibition percentage of nitroxyl radical by the sample and was calculated using the formula [38, 39]

$$RSA(\%) = \frac{(I_0 - I_t)}{I_0} \times 100$$

where I_0 is the signal intensity of central line from the ESR spectrum, when nitroxyl radicals in pure water, I_t is the signal intensity of central line from the ESR spectrum, when nitroxyl radicals in ascorbic acid at time t. Fig. 5b

shows the radical scavenging activity (%) of 1mM concentration of ^{14}N-labeled TEMPONE, TEMPO and TEMPOL in 1mM concentration of ascorbic acid at various time intervals. The observed radical scavenging activity is higher for TEMPONE radical compared with TEMPO and TEMPOL radicals.

4. Conclusions

The ESR parameters such as the line width, g-factor, hyperfine coupling constant and rotational correlation time for 1 mM concentration of ^{14}N-labeled TEMPONE, TEMPO and TEMPOL in pure water were obtained. From the results, the TEMPONE radical has narrowest line width and fast tumbling motion compared with TEMPO and TEMPOL radicals. The reduction process was recorded for 1mM concentration of ^{14}N-labeled TEMPONE, TEMPO and TEMPOL in 1mM concentration of ascorbic acid as a function of time using X- band ESR spectrometer. The half life time and decay rate were estimated for 1mM concentration of ^{14}N labeled nitroxyl radicals in 1 mM concentration of ascorbic acid. From the results, the increase in half life time and decrease in decay rate were calculated for TEMPONE compared with TEMPO and TEMPOL radicals, which indicates the higher stability of TEMPONE radical. The observed radical scavenging activity is also higher for TEMPONE radical. Hence, the TEMPONE radical can act as a good redox sensitive spin probe for in vivo/in vitro ESR and OMR Imaging.

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