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Comparative Study of the Reduction Process of Different Ring Structured Nitroxyl Spin Probes: An Electron Spin Resonance Study

V. Meenakumari[1], A. Jawahar[2], A. Milton Franklin Benial[1, *]

[1]Department of Physics, NMSSVN College, Nagamalai, Madurai, Tamilnadu, India

[2]Department of Chemistry, NMSSVN College, Nagamalai, Madurai, Tamilnadu, India

Email address:

meenakumariv78@gmail.com (V. Meenakumari), ajanthajawahar@yahoo.com (A. Jawahar), miltonfranklin@yahoo.com (A. M. F. Benial)

[*]Corresponding author

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Abstract: Electron spin resonance spectroscopy (ESR) studies on the reduction process of nitroxyl spin probes were carried out for 1mM concentration of ^{14}N-labeled pyrrolidine nitroxyl spin probes, 3-carbamoyl-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (carbamoyl-PROXYL) and 3-carboxy-2,2,5,5-tetramethyl-pyrrolidine -1-oxyl (carboxy-PROXYL), ^{14}N-labeled piperidine nitroxyl spin probes, 4-methoxy-2,2,6,6-tetramethyl-piperidine-1-oxyl (methoxy-TEMPO) and 4-acetamido-2,2,6,6-tetramethyl-piperidine-1-oxyl (acetamido-TEMPO) in 1 mM concentration of ascorbic acid as a function of time. The half-life time and decay rate were estimated. The piperidine nitroxyl spin probes show a short half-life time compared with that of pyrrolidine nitroxyl spin probes. This result indicates that the higher stability of pyrrolidine nitroxyl spin probes. The ESR was also recorded for 1mM concentration of pyrrolidine nitroxyl spin probes and piperidine nitroxyl spin probes in pure water using X-band ESR spectrometer. The ESR parameters such as line width, hyperfine coupling constant, g-factor, signal intensity ratio and rotational correlation time were determined. These results indicate that the pyrrolidine nitroxyl spin probes have narrow line width and fast tumbling compared with the piperidine nitroxyl spin probes. Therefore, this study reveals that the pyrrolidine nitroxyl spin probes can act as good redox sensitive spin probes for free radical imaging.

Keywords: Electron Spin Resonances (ESR), Nitroxyl Spin Probe, Ascorbic Acid, Half Life Time, Decay Rate

1. Introduction

The nitroxyl radicals are well-known stable organic radicals. Most frequently used nitroxyl radicals *invivo* are piperidine and pyrrolidine classes. The stability of nitroxyl radicals is attributed to the three π-bonding system with a delocalized unpaired electron on the N-O bond. These nitroxyl radicals are less toxic, and thereby preferable for *invivo* applications. Nitroxyl radicals are susceptible to oxygen concentration [1], reactive oxygen species (ROS) [2-3], and biological redox systems [4-8], and are widely used as probes for invivo electron spin resonance (ESR) measurement [9-11]. The most active use of *invivo* ESR spectroscopy and its imaging has been in the measurement of redox status, radical generation and partial pressure of oxygen. ESR spectroscopy has been employed in *invitro* redox research studies [12, 13]. In the ESR X-band frequency 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 [14].

The reduction of nitroxyl radicals by cell is primarily intracellular [15]. Ascorbic acid plays a significant role in the nitroxyl radical reduction in erythrocytes, hepatocytes, and kidney cells, which are rich in this compounds [16, 17]. Ascorbic acid is one of the primary reducing agent in biochemistry, which is directly involved in many biochemical processes, such as metabolic-oxidation-reduction, carcinogensis, and aging. The paramagnetic nitroxyl radicals could be reduced to diamagnetic hydroxylamine by

antioxidants, such as ascorbic acid (vitamine C) or enzymatic system, with a loss of ESR signal and thus serves as a reduction sensor. However, the diamagnetic hydroxylamine could be reconverted via oxygenation to paramagnetic nitroxyl radicals with an appearance of ESR signal and thus serves as an oxidation sensor. The rate constant of both processes could be used for evaluation of reduction / oxidation balance in cells and tissues, using ESR imaging or magnetic resonance imaging (MRI) [18]. The mechanism of reduction in *invivo* experiments depends on several factors including the structure of the nitroxyl radical, oxygen concentration, membrane permeability, extracellular reduction by ascorbate, and possible reoxidation of the reduced form by nitroxyl radicals. The redox properties of nitroxyl radicals can give us double benefits which are (i) as redox-sensitive contrast agents and (ii) as a normal tissue-selective radio-protector. Both benefits are useful for a first diagnosis of a tumor and radiation therapy planning. These will give a radio-protective treatment for normal tissues in subsequent radiotherapy. Therefore, the magnetic resonance based redox imaging techniques using nitroxyl contrast agents can have wide possibility.

Earlier studies have shown that a cell-permeable nitroxide was reduced faster in tumor than in normal tissue [19, 20]. Therefore, monitoring the rate of transformation of suitable nitroxides to the corresponding diamagnetic species using MRI can provide *invivo* assessment of redox status in a noninvasive manner. Utsumi et al. obtained the simultaneous molecular imaging of reduction and oxidation processes monitored by overhauser magnetic resonance imaging (OMRI) with ^{14}N-and ^{15}N-labeled nitroxyl radicals [21]. Benial et al. reported that the permeability studies of redox sensitive nitroxyl spin probes through lipid membranes using an L-band ESR spectrometer [22]. Kinoshita et al. also reported the synthesis of tetraethyl-substitued nitroxyl radicals, which exhibit remarkable resistivity towards ascorbic acid reduction during *invivo* testing [23].

Recently, the stable nitroxyl radicals have been used as redox sensitive probe for *invivo* ESR and OMRI techniques [24-34]. In order to understand the reduction process and find the suitable nitroxyl spin probe among the pyrrolidine and piperidine nitroxyl radicals for *invivo/invitro* ESR and OMRI techniques. Here, we report the ESR spectroscopy studies on the kinetics and reduction mechanism of 1mM concentration of ^{14}N-labeled carbamoyl-PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in pure water and 1 mM concentration of ascorbic acid.

2. Materials and Methods

2.1. Chemicals

The spin probes, 3-carbamoyl-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (carbamoyl-PROXYL), 3-carboxy -2,2,5,5-tetramethyl-pyrrolidine-1-oxyl-(carboxy-PROXYL), 4-methoxy-2,2,6,6-tetramethyl-piperidine-1-oxyl (methoxy-TEMPO), 4-acetamido-2,2,6,6-tetramethyl-piperidine-1-oxyl (acetamido-TEMPO) and ascorbic acid were purchased from Sigma Aldrich Chemical Co, St. Louis, MO, USA. Deionized water (deionization by the Milli-Q system) was used for this experiment.

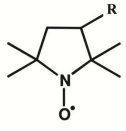
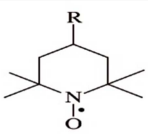
2.2. ESR Measurements

ESR spectra were recorded for 1 mM concentration of ^{14}N-labeled carbamoyl-PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in pure water and 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 k; and microwave frequency, 9.86 GHz. The ESR spectra were recorded in the first derivative mode at 27°C. The temperature was controlled using a controller with water as a coolant. The ESR line width measurements were carried out for 1 mM concentration of nitroxyl spin probes in pure water with an accuracy of ~±0.5 μT.

3. Results and Discussion

The schematic diagram of the reduction process of nitroxyl spin probe is shown in Figure 1. The ESR spectra of 1mM concentration of ^{14}N-labeled carbamoyl- PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in pure water and 1 mM concentration of ascorbic acid as a function of time are shown in Figures 2-5. The signal intensity and scavenging activity of all nitroxyl spin probes in pure water and 1 mM concentration of ascorbic acid at various time intervals are shown in Figures 6-7. The ring structures and abbreviations of nitroxyl spin probes are shown in Table 1. The ESR parameters such as line width, hyperfine coupling constant, g-factor, signal intensity ratio and rotational correlation time for 1mM concentration of ^{14}N-labeled carbamoyl- PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in pure water are listed in Table 2. The half life time and decay rate of 1mM concentration of ^{14}N-labeled carbamoyl-PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in 1mM concentration of ascorbic acid are listed in Table 3.

Table 1. *The Ring structures and abbreviations of nitoxyl spin probes.*

Basic structure	Substituents (R)	Abbreviations
	$2CONH_2$	carbamoyl-PROXYL
	2COOH	carboxy-PROXYL
	OCH_3	methoxy-TEMPO
	$NHCOCH_3$	acetamido-TEMPO

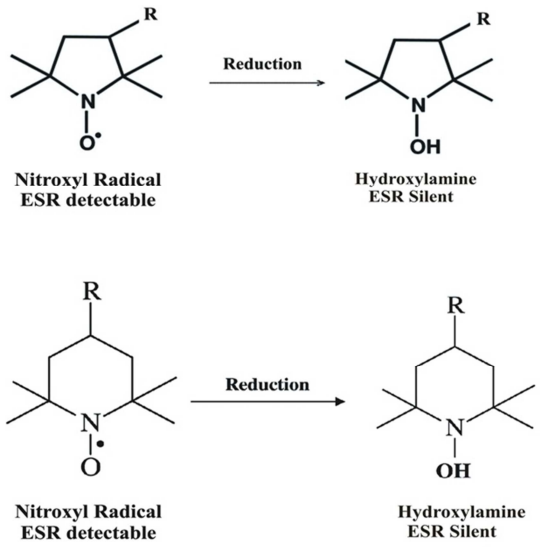


Figure 1. *Schematic diagram of the reduction process of nitroxyl spin probes, R represents substituents.*

3.1. Line Width

The ESR line width values for 1 mM concentration of ^{14}N-labeled carbamoyl-PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in pure water were calculated and listed in Table 2. The line width broadening is due to the dipolar and spin exchange interactions of agent concentrations. The line width broadening is due to the interaction between the subtituents (R) in pyrrolidine, piperidine nitroxyl spin probes and water proton. These results are in good agreement with the previous study [35-37]. The pyrrolidine nitroxyl spin probes viz., carbamoyl-PROXYL and carboxy-PROXYL have a narrow line width compared with the piperidine nitroxyl spin probes viz., methoxy-TEMPO and acetamido-TEMPO. The narrow line width values of pyrrolidine nitroxyl spin probes indicate that the interaction between the carbamoyl group/carboxyl group of pyrrolidine nitroxyl spin probes and water proton is less, but the interaction between the methyl group of piperidine nitroxyl spin probes and water proton is more. The observed line width value is ~42% higher for piperidine nitroxyl spin probes compared with the pyrrolidine nitroxyl spin probes.

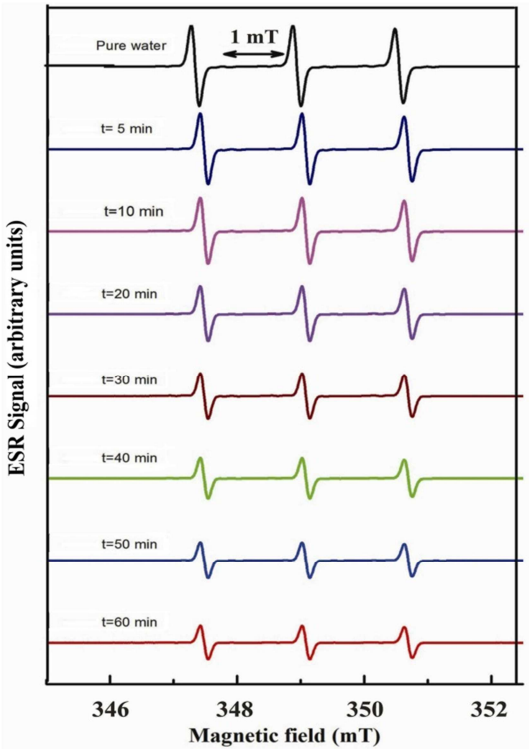


Figure 2. *ESR spectra of 1mM concentration of ^{14}N-labeled carbamoyl-PROXYL in pure water and 1mM concentration of ascorbic acid as a function of time (t).*

Table 2. *Electron spin resonance parameters for 1mM concentration of ^{14}N-labeled carbamoyl-PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO 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) ($\times 10^{-11}$)
carbamoyl-PROXYL	117.30	1.603	2.0117	1.0842	3.145
carboxy-PROXYL	119.03	1.603	2.0118	1.0879	3.336
methoxy-TEMPO	166.07	1.701	2.0103	1.0675	3.584
acetamido-TEMPO	166.18	1.706	2.0109	1.0931	4.918

3.2. Hyperfine Coupling Constant and g-factor

The Hyperfine coupling constant and g-factor values for 1 mM concentration of ^{14}N-labeled carbamoyl-PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in pure water are listed in Table 2. The observed hyperfine coupling constant is less for pyrrolidine nitroxyl spin probes compared with piperidine nitroxyl spin probes. This shows that the Fermi contact interaction is less for pyrrolidine nitroxyl spin probes viz., carbamoyl-PROXYL and carboxy-PROXYL compared with piperidine nitroxyl spin probes viz., methoxy-TEMPO and acetamido-TEMPO. The hyperfine coupling constant of the nitroxyl spin probes agree well with the previous study [35-37]. The obtained g-factor value confirms the isotropic nature of the system.

3.3. Signal Intensity Ratio

The ESR signal intensity ratio for 1 mM concentration of ^{14}N-labeled carbamoyl-PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in pure water are listed in Table 2. The ESR signal intensity ratio becomes the unity for both pyrrolidine and piperidine nitroxyl spin probes in pure water, which reveals that the homogenous nature of the samples. These results agree well with the reported values [35-37].

Table 3. *The half life time and decay rate of ^{14}N-labeled carbamoyl PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in 1mM concentration of ascorbic acid*

Sample	Half life time $t_{1/2}$ (min)	Signal decay rate $1/\tau$ (min^{-1})
carbamoyl-PROXYL	17.801	0.039
carboxy-PROXYL	13.353	0.052
methoxy-TEMPO	4.937	0.140
acetamido-TEMPO	2.644	0.262

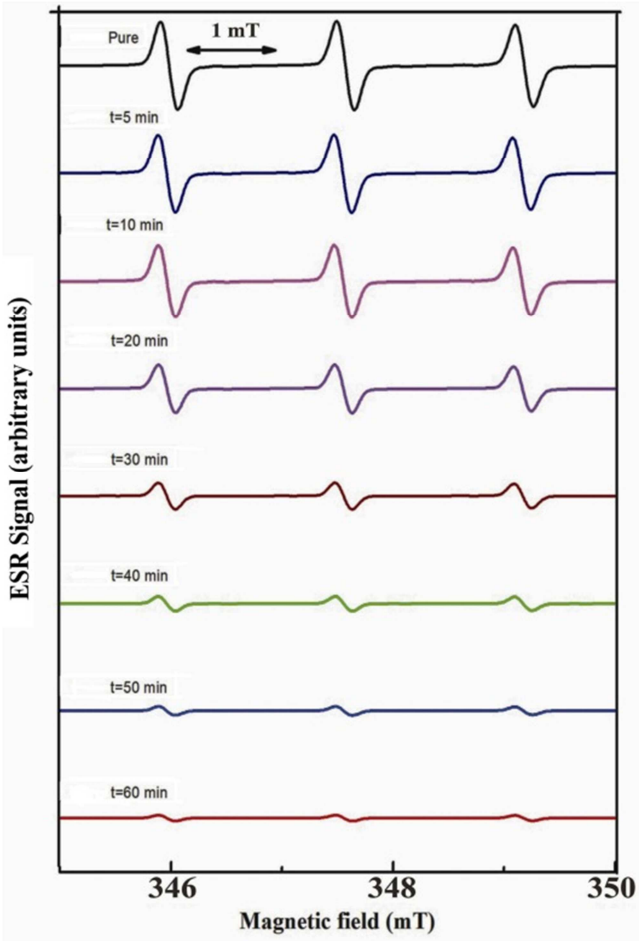


Figure 3. *ESR spectra spectra of 1mM concentration of ^{14}N-labeled carboxy-PROXYL in pure water and 1mM concentration of ascorbic acid as a function of time (t).*

3.4. Rotational Correlation Time

The rotational correlation time describes the dynamics of the spin probe motion in the domain and it is proportional to the fluidity. The rotational correlation time is a parameter to express the mobility of spin probes in their environment. The τ_R can be obtained from the ESR spectral line width and relative intensities. The rotational correlation time is given by an empirical formula

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

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 [38, 39]. The rotational motion of the spin probe was assumed to be isotropic.

The rotational correlation time for 1 mM concentration of ^{14}N-labeled carbamoyl-PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in pure water were calculated and listed in Table 2. The decrease in rotational correlation time was observed for pyrrolidine nitroxyl spin probes, carbamoyl-PROXYL and carboxy-PROXYL compared with the piperidine nitroxyl spin probes, methoxy-TEMPO and acetamido-TEMPO in pure water. These results show that the pyrrolidine nitroxyl spin probes has fast tumbling motion compared with the piperidine nitroxyl spin probes. Among the pyrrolidine nitroxyl spin probes, the carbaomyl-PROXYL has fast tumbling motion which is due to the less interaction between the carbonyl group of carbamoyl-PROXYL radical and water protons. The narrowest line width observed is an additional supporting evidence of the fast tumbling motion of the pyrrolidine nitroxyl spin probes.

3.5. Reduction Stability

The paramagnetic nitroxyl radical can be reduced to the corresponding hydroxylamines by reductants such as ascorbic acid. Paramagnetic nitroxyl radicals can be directly detected by ESR, while the hydroxylamines which are the reduction products of nitroxyl radicals are diamagnetic and therefore ESR silent species.

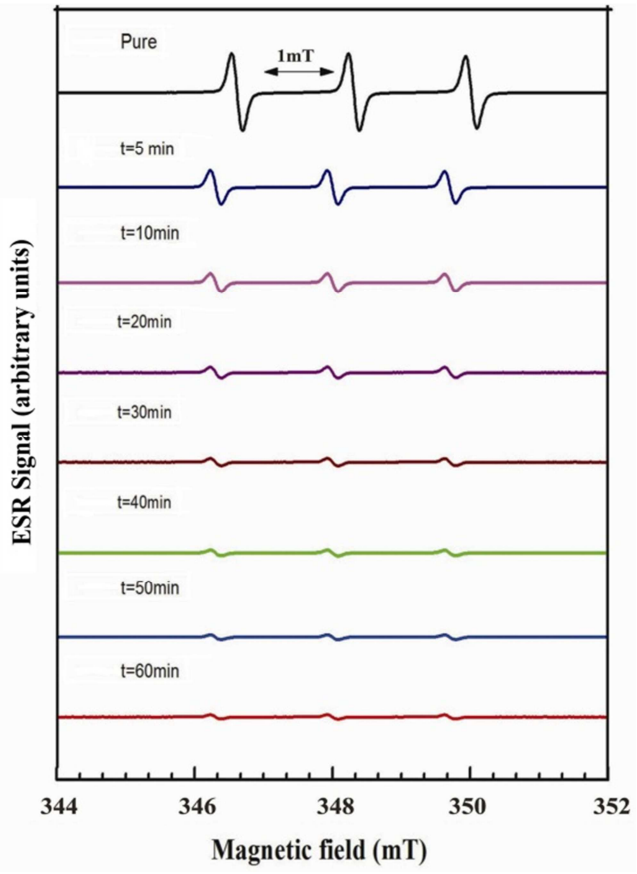


Figure 4. *ESR spectra of 1mM concentration of ^{14}N-labeled methoxy-TEMPO in pure water and 1mM concentration of ascorbic acid as a function of time (t).*

Therefore, detecting a decrease in ESR signal intensity from nitroxyl radicals due to their reduction in biological/chemical samples can be used to monitor redox *invivo* reactions [40, 41, 25-28]. The ESR spectrum of 1mM concentration of ^{14}N-labeled carbamoyl PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in pure water and 1 mM concentration of ascorbic acid as a function of time are shown in Figures 2-5. The intensity of the ESR signal is strongly reduced in the presence of the ascorbic acid. The reduction in the signal intensity is time dependent. The reduction rate by ascorbic acid is dependent mainly upon their ring structures and substituent groups. The signal decay of several spin probes with different ring structures and substituents were compared.

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 [42, 43]. 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. The decay curve for 1mM concentration of ^{14}N-labeled carbamoyl-PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in pure water and 1 mM concentration of ascorbic acid was shown in Figure 6.

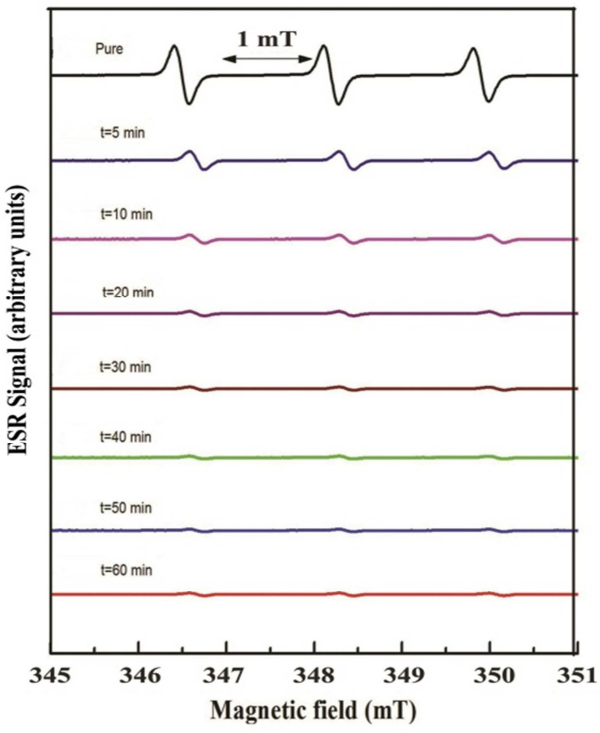


Figure 5. *ESR spectra of 1mM concentration of ^{14}N-labeled acetamido-TEMPO in pure water and 1mM concentration of ascorbic acid as a function of time (t).*

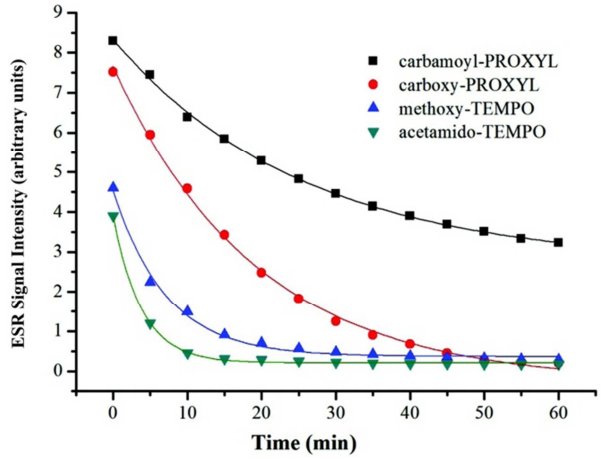


Figure 6. *Time dependent decay of the ESR signal intensity for 1mM concentration of ^{14}N-labeled carbamoyl PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in pure water and 1mM concentration of ascorbic acid.*

The correlation coefficient of the above curve fitting shows the best correlation (R^2>0.99). The half-life time can be calculated by using the equation,

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

The half-life time and decay rate were calculated for 1mM concentration of ^{14}N-labeled carbamoyl PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in 1 mM concentration of ascorbic acid and listed in Table 3. The increase in half life time and decrease in decay rate were obtained for pyrrolidine nitroxyl spin probes compared with piperidine nitroxyl spin probes, which shows the higher stability of pyrrolidine nitroxyl spin probes. Among the pyrrolidine nitroxyl spin probes, the carbamoyl-PROXYL can act as a good permeable spin probe for *invivo* free radical imaging. The carboxy-PROXYL can act as a good impermeable spin probe for *invivo* free radical imaging.

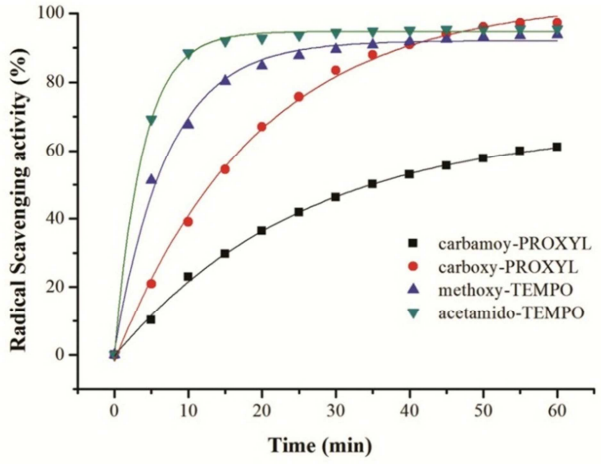


Figure 7. *The radical scavenging activity (%) of 1mM concentration of ^{14}N-labeled carbamoyl PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in pure water and 1mM concentration of ascorbic acid as a function of time.*

3.7. Radical Scavenging Activity

Radical scavenging activity (RSA) was expressed as the inhibition percentage of nitroxyl spin probes and calculated using the formula

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

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 spin probes in ascorbic acid at time t [44, 45]. Figure 7 shows the radical scavenging activity (%) of 1mM concentration of ^{14}N-labeled carbamoyl-PROXYL, carboxy-PROXYL, methoxy-TEMPO and acetamido-TEMPO in 1mM concentration of ascorbic acid at various time intervals. The observed radical scavenging activity is higher for pyrrolidine nitroxyl spin probes compared with the piperidine nitroxyl spin probes.

4. Conclusions

The ESR reduction process was recorded for nitroxyl spin probes of different ring structure with ascorbic acid as a function of time using X-band ESR spectrometer. The half-life time and decay rate were estimated. From the results, the increase in half life time and decrease in decay rate were obtained for pyrrolidine nitroxyl spin probes compared with the piperidine nitroxyl spin probes, which indicates the higher stability of pyrrolidine nitroxyl spin probes. The ESR spectra was also recorded for 1mM concentration of nitroxyl spin probes in pure water. The ESR parameters such as the line width, g-factor, hyperfine coupling constant and rotational correlation time were estimated. From the results, the pyrrolidine nitroxyl spin probes have narrow line width and fast tumbling motion compared with the piperidine nitroxyl spin probes. Hence, the pyrrolidine nitroxyl spin probes viz., carbamoyl-PROXYL can act as a good permeable spin probe and carboxy-PROXYL can act as a good impermeable spin probe for *invivo* free radical imaging. The reduction of nitroxyl spin probes could be useful as indicator of *invivo* redox metabolism.

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