CHAPTER-VI

ROTATIONAL CORRELATION TIME STUDIES ON NITROXYL RADICALS USING 300 MHz ESR SPECTROMETER IN HIGH VISCOUS LIQUID

ABSTRACT

The mobility studies on ^{14}N-labeled TEMPONE, TEMPO, carbamoyl-PROXYL, carboxy-PROXYL in the high viscous liquid was carried out using 300 MHz electron spin resonance (ESR) spectrometer. The ESR parameters, such as line width, signal intensity ratio, g-factor, hyperfine coupling constant and correlation time were determined. The line width broadening increases twofold in high viscous samples of ^{14}N-labeled carbamoyl-PROXYL and carboxy-PROXYL, but this line broadening is negligibly small in the high viscous sample (85% glycerol) of ^{14}N-labeled TEMPO. The correlation time also increases (~30 times) in the high viscous sample (85% glycerol) of ^{14}N-labeled carbamoyl-PROXYL and carboxy-PROXYL, but there is no considerable increase in the high viscous sample of ^{14}N-labeled TEMPO. TEMPONE has the narrowest line width and is also highly sensitive to viscosity. The correlation time increases (~13 times) in the high viscous sample (85% glycerol) of ^{14}N-labeled TEMPONE. Therefore, this study reveals that the ^{14}N-labeled TEMPONE radical is the most suitable spin probe for in vivo/in vitro studies in high viscous biological fluids.

6.1 INTRODUCTION

Nitroxyl-free radicals are of particular interest from biological point of view. In vivo studies have not yet revealed any serious toxicity problems when nitroxides are injected intravenously [1–5] and they may be attached to other biomolecules [6–9], making them particularly suitable to targeting studies. Nitroxyl radicals have been widely used as spin probes for low-frequency in vivo electron spin resonance imaging (ESRI), and as contrast agents for Overhauser magnetic resonance imaging (OMRI) [10-12]. Lurie et al. [13] used nitroxyl radicals and successfully obtained images of tissue water protons in the vicinity of paramagnetic radicals and so achieved in vivo imaging. By isotopically labeling nitroxyl spin probes, we have recently investigated their application as redox-sensitive molecular probes for simultaneous molecular imaging by OMRI [10,14]. ESRI and OMRI techniques are powerful biomedical tools for in vivo free radical imaging. The involvement of free radicals in a range of diseases has provoked considerable interest in the development of free radical imaging [10–21]. The ESRI and OMRI intensities correlated with the ESR parameters. Recent studies revealed that the ESR parameters depend on physical and chemical properties of free radicals [19]. The dynamic nuclear polarization (DNP) studies of redox-sensitive nitroxyl spin probes in liposomal solution reveals that OMRI can be used to differentiate between intra and extra membrane water by loading the liposome vesicles with a lipid-permeable nitroxyl spin probe [22]. The permeable and impermeable nitroxyl radicals are receiving increased attention in ESRI and OMRI as viable reporter of in vivo redox status. Simultaneous monitoring of the spin probe in aqueous and lipid phases is necessary to arrive at the in vivo redox status [23]. Using lipid-permeable and impermeable nitroxyl spin probes,

one can monitor intra- or extra cellular redox status. The DNP parameters relevant to OMRI were reported for nitroxyl radicals used as spin probes in imaging [14,24-27]. These DNP parameters depend on the ESR parameters, such as line width, line shape, hyperfine constant, g-factor and rotational correlation time. The OMRI studies revealed that the dependence of image intensity on the proton mobility within biological samples may provide an indicator of the pathological state of the sample [24]. In order to study the molecular dynamics in biological samples, such as high-viscosity fluid plasma membrane, the present ESR study was carried out in high-viscosity liquid mixture (pure water/glycerol) using 300 MHz ESR spectrometer. In this chapter, the ESR study of ^{14}N-labeled TEMPONE, TEMPO, carbamoyl-PROXYL and carboxy-PROXYL in pure water and pure water/glycerol mixture in the ratio of 15:85 is presented.

6.2 MATERIALS AND METHODS

The spin probes, 4-oxo-2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPONE), 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO), 3-carbamoyl-2,2,5,5-tetramethyl-pyrrolidine-1-yloxy (carbamoyl-PROXYL), 3-carboxy-2,2,5,5,-tetramethyl-1-pyrrolidinyloxy (carboxy-PROXYL) were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA).

6.2.1. ESR Measurements

The ESR measurements were carried out 300 MHz ESR spectrometer with a loop gap resonator (internal diameter, 32 mm; length, 28 mm). The loop-gap resonator is used for in vivo ESR measurements [23]. The ESR spectra were recorded for 2 mM concentration of ^{14}N-labeled TEMPONE, TEMPO, carbamoyl-PROXYL and carboxy-PROXYL in pure water and pure water/ glycerol mixture in the ratio of 15:85 by varying the magnetic field in the range

of 8–13.5 mT. The ESR acquisition parameters were as follows: modulation frequency, 12 kHz; field modulation amplitude, 0.1 mT; time constant, 300 ms; radio-frequency power, 4 mW; sweep width, 6 mT and radio frequency, 300 MHz. The ESR spectrum was recorded in the first-derivative mode at 27˚C. To remove oxygen from the solution, argon gas was passed through the samples for about 2 h. The samples were prepared using the phosphate buffer solution at pH 7.4. The samples with pure water and pure water/glycerol mixture were loaded into an ESR phantom with diameter of 2.8 cm. For each measurement, the phantom was filled with samples 4 cm long with volume of 20 ml. The temperature was controlled using a controller with water as a coolant.

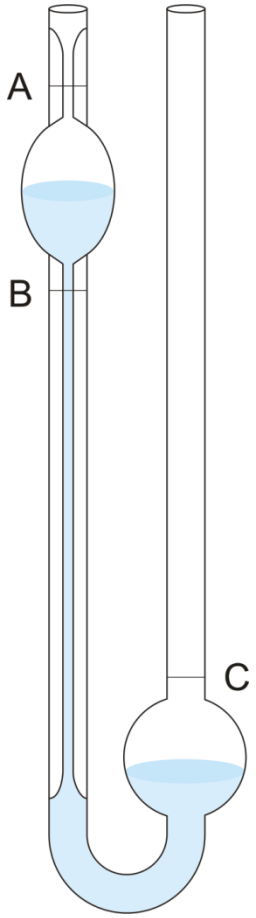

Fig. 6.1 U-tube Viscometer

6.2.2 Viscosity Measurements

The viscosity was measured at room temperature using Ostwald's viscometer (Fig. 6.1) to an accuracy of ±0.2%. The density measurements were made using a precalibrated 5 ml specific gravity bottle to an accuracy of ±2 parts in $10^{4.}$ The viscosity of the solution was determined using the following equation

$$\eta_s = (\rho_s t_s / \rho_w t_w) \eta_w \qquad (6.1)$$

where η_s is the viscosity of the sample, η_w is the viscosity of water, ρ_w is the density of the water, ρ_s is the density of the sample, t_w is the time taken for water to flow from the mark A to B in the viscometer, t_s is the time taken for the sample to flow from the mark A to B in the viscometer.

6.3 RESULTS AND DISCUSSION

6.3.1 Line width

The ESR spectra for 2 mM ^{14}N-labeled TEMPONE, TEMPO carbamoyl-PROXYL and carboxy-PROXYL in pure water and pure water/glycerol mixture in the ratio of 15:85, which are shown in Figs. 6.2–6.5. The ESR line width values are given in Table 6.1. The line width broadening is due to the dipolar and spin exchange interactions of agent concentrations. These results agree well with the previous studies [14,24,25,28]. Table 6.1 shows that the line width broadening increases in the samples, pure water/glycerol mixture in the ratio of 15:85. The line width broadening increases two fold in the high-viscosity samples (85% glycerol) of ^{14}N-labeled carbamoyl-PROXYL and carboxy-PROXYL, but this effect is significantly smaller in the high-viscosity sample (85% glycerol) of ^{14}N-labeled TEMPO. The line width broadening increases almost by 50% in the high-viscosity samples (85% glycerol) of ^{14}N-labeled TEMPONE. Hence, the sensitivity of

TEMPO radical is significantly smaller when compared with the other nitroxyl radicals. However, the TEMPONE radical has two important factors: the narrowest line width and high sensitivity. Among the four nitroxyl radicals, TEMPONE, carbamoyl-PROXYL, and carboxy-PROXYL have a carbonyl group. This carbonyl group interacts with the water proton and glycerol OH protons. Therefore, this interaction leads to the line broadening mechanism in the high-viscosity medium (pure water/glycerol). However, the carbonyl group is not present in the TEMPO molecule. Hence the interaction of TEMPO with water protons and glycerol OH protons is very small. Therefore, the line width broadening is negligibly small in the high-viscosity liquid mixture (pure water/glycerol) compared with the other nitroxyl radicals.

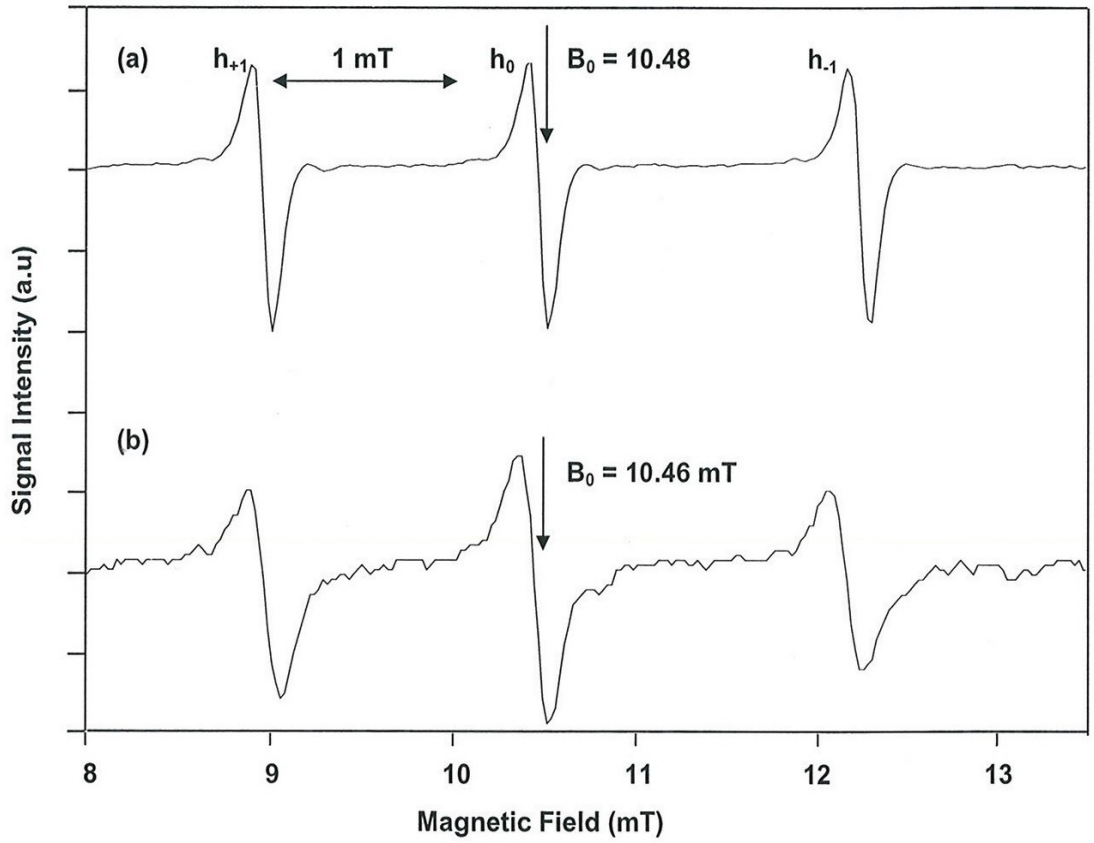

Fig. 6.2 **(a)** ESR spectrum of 2 mM ^{14}N labeled TEMPONE in pure water
(b) ESR spectrum of 2 mM ^{14}N labeled TEMPONE in pure water/glycerol mixture in the ratio of 15:85

6.3.2. Hyperfine Coupling Constant

Table 6.1 shows that the average hyperfine coupling constant and g-factor for ^{14}N-labeled TEMPONE, TEMPO, carbamoyl-PROXYL and carboxy-PROXYL. The hyperfine coupling constants for 2 mM ^{14}N-labeled TEMPONE, TEMPO, carbamoyl-PROXYL and carboxy-PROXYL agree well with the previous study [14]. There is an appreciable change in the average hyperfine coupling constant for 2 mM ^{14}N-labeled TEMPONE, carbamoyl-PROXYL and carboxy-PROXYL in the high viscous liquid medium (85% glycerol), but there is no significant change for 2 mM ^{14}N-labeled TEMPO in the high-viscosity liquid mixture (pure water/glycerol), which indicates that the Fermi contact interaction term remains the same in high viscous liquid medium.

6.3.3. Rotational Correlation Time

The electron spin resonance (ESR) spectroscopy is a powerful technique to obtain information about the rotational mobility of spin-labeled molecules, because a very broad range of molecules can be covered by this technique. The molecular mobility has been studied using ESR spectroscopy by labeling polymers and food materials with a suitable spin probe [29]. From the ESR spectra of the spin probe, τ_R can be assessed [30]. The τ_R values (10^{-12} to 10^{-9} s) can be calculated from conventional ESR spectra [31]. The saturation transfer (ST)-ESR spectroscopy technique further expands this range to very slow 10^{-6} to 10^{-3} s [32] and ultra slow molecular motions [29]. ST-ESR spectroscopy has been used to determine the τ_R values of spin probes in glassy sugar–water systems [33]. Lucaciu et al. [34] carried out the ESR study of the dynamic behavior of spin labels (TEMPO and TEMPONE) inclusion in cyclodextrins. Recently, ESR studies on the dependence of the radical concentration and the

solvent viscosity has been reported [35]. Conventional ESR spectroscopy can detect changes in τ_R of spin probes ranging from 10^{-12} to 10^{-9} s, which correspond to the lifetime of the probe in the given orientation. In this motional range, the ESR spectrum of nitroxyl radical consists of three lines, and τ_R can be calculated by the method of Knowles et al. [30,31].

$$\tau_R = 6.5 \times 10^{-10} \Delta B_0 [(h_0/h_{-1})^{1/2}-1] \qquad (6.2)$$

where h_{-1} and h_0 are the amplitudes of the high-field and central lines in the ESR spectra, 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.

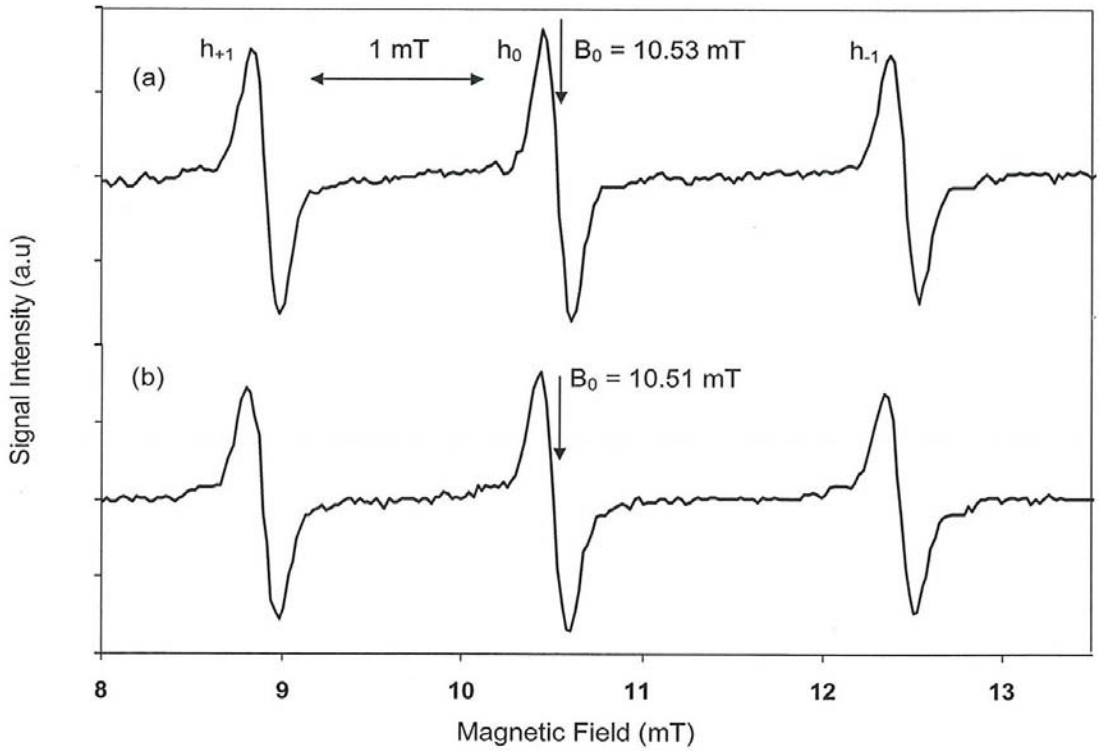

Fig. 6.3 **(a)** ESR spectrum of 2 mM ^{14}N labeled TEMPO in pure water

(b) ESR spectrum of 2 mM ^{14}N labeled TEMPO in pure water and pure water/glycerol mixture in the ratio of 15:85

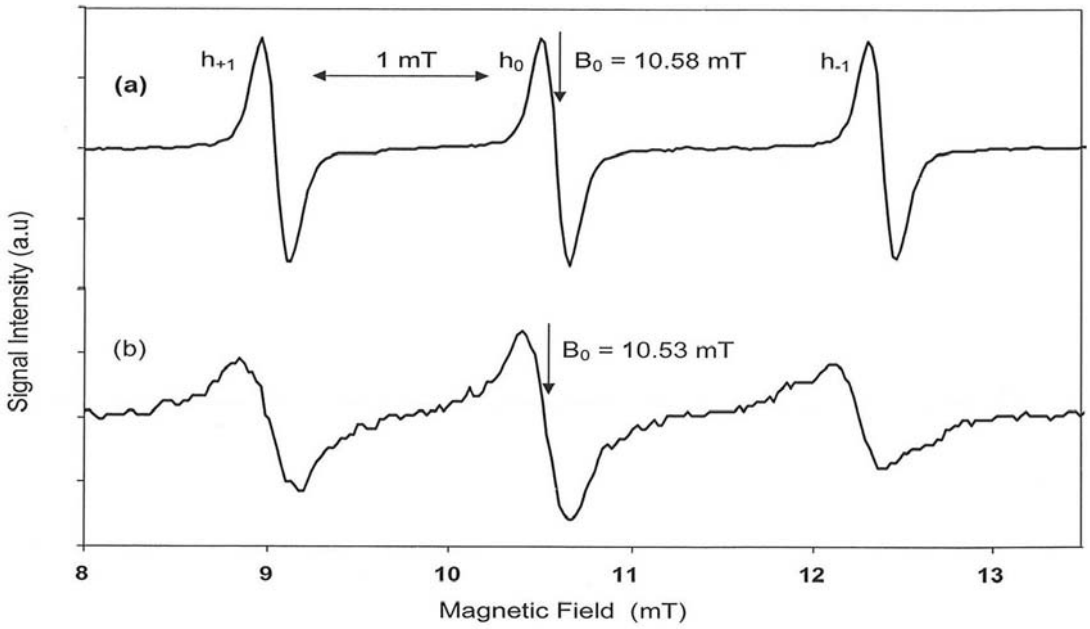

Fig. 6.4 **(a)** ESR spectrum of 2 mM ^{14}N labeled carbamoyl-PROXYL in pure water **(b)** ESR spectrum of 2 mM ^{14}N labeled carbamoyl-PROXYL in pure water/glycerol mixture in the ratio of 15:85

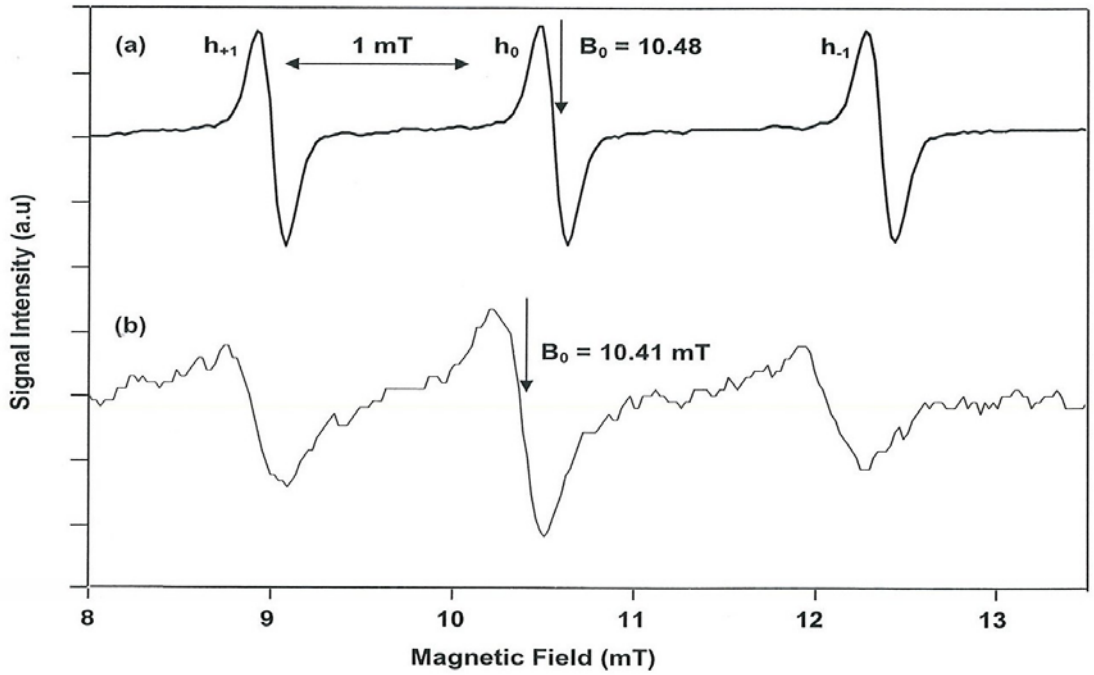

Fig. 6.5 **(a)** ESR spectrum of 2 mM ^{14}N labeled carboxy-PROXYL in pure water, **(b)** ESR spectrum of 2 mM ^{14}N labeled carboxy-PROXYL in pure water/glycerol mixture in the ratio of 15:85

Table 6.1 ESR parameters of nitroxyl radicals in pure water, pure water/ glycerol mixture in the ratio of 15:85

Samples		signal intensity ratio h_0/h_{-1}	linewidth ΔB (μT)	g-value	A_{iso} (mT)	correlation time τ_R (s) ($\times 10^{-11}$)
TEMPONE	Pure water	1.05	95	2.0334	1.640	1.49
	15:85* (ratio)	1.50	140	2.0477	1.582	20.4
TEMPO	Pure water	1.18	164	2.0340	1.769	9.19
	15:85* (ratio)	1.19	175	2.0390	1.757	10.33
carbamoyl-PROXYL	Pure water	1.04	140	2.0206	1.670	1.80
	15:85* (ratio)	1.82	281	2.0340	1.640	63.8
carboxy-PROXYL	Pure water	1.04	140	2.0206	1.675	1.80
	15:85* (ratio)	1.76	269	2.0340	1.593	65.8

∗indicates the viscosity of the mixture is 54.73 cP

The rotational correlation time for 2 mM concentration of ^{14}N-labeled TEMPONE, TEMPO, carbamoyl-PROXYL and carboxy-PROXYL in pure water and pure water/glycerol mixture in the ratio of 15:85 are given in Table 6.1. The rotational correlation time agrees well with the previous study [31]. The rotational correlation time for 2 mM ^{14}N-labeled TEMPO in pure water was estimated as 9.19 x 10^{-11} s, whereas for 2 mM ^{14}N-labeled carbamoyl-

PROXYL and carboxy-PROXYL in pure water, the rotational correlation time was estimated as 1.80 x 10^{-11} s. These results show that the tumbling motion of the nitroxyl radical is very slow in 2 mM ^{14}N-labeled TEMPO solution in pure water samples. This result reveals that the ESR behavior is different for TEMPO radical. The rotational correlation time increases (~30 times) for 2 mM ^{14}N-labeled carbamoyl-PROXYL and carboxy-PROXYL solution in 85% glycerol (54.7 cP), but there is no significant change noticed for ^{14}N-labeled TEMPO in 85% glycerol, which reveals that the mobility of nitroxyl radical is not restricted by the high viscous liquid medium. The rotational correlation time increases (~13 times) for 2 mM ^{14}N-labeled TEMPONE in the high-viscosity liquid mixture (pure water/glycerol).

6.3.4. ESR signal intensity ratio

The ESR signal intensity ratio value becomes unity for the pure water samples. The signal intensity ratio increases with increasing viscosity. There is an appreciable change in the high-viscosity medium for ^{14}N-labeled TEMPONE, carbamoyl- PROXYL and carboxy-PROXYL radicals. Table 6.1 shows that the signal intensity ratio is very high for ^{14}N-labeled TEMPONE, carbamoyl-PROXYL and carboxy-PROXYL in 85% glycerol, but there is no significant change for TEMPO solution in 85% glycerol, which shows that the system behaves as an isotropic in high viscous medium.

6.3.5. DNP Parameters

The Overhauser enhancement reaches maximum for pure dipolar and scalar interactions. These maximum values are not realized due to many experimental factors [14]. To achieve the maximum Overhauser enhancement factor, the nitroxyl spin probe should have a narrow ESR line width. However, the three ESR lines are inhomogeneously broadened in the high-viscosity medium. Hence, the irradiation of one of the ESR lines will result only in

partial saturation. This partial saturation leads to the reduction in the signal intensity enhancement. Therefore, the saturation parameter is an important DNP parameter for obtaining the OMRI. For achieving the maximum saturation, OMRI needs narrow line width spin probe in the high-viscosity medium. This ESR study reveals that the TEMPONE radical can be used as the narrow line width sensitive spin probe for OMRI in the high-viscosity samples.

6.4 CONCLUSIONS

The ESR parameters of the line width, signal intensity ratio, hyperfine coupling constant, and rotational correlation time were reported for 2 mM ^{14}N-labeled TEMPONE, TEMPO, carbamoyl-PROXYL and carboxyl-PROXYL in pure water and pure water/glycerol mixture in the ratio of 15:85. The line width broadening increases two fold in the high-viscosity sample (85% glycerol) of ^{14}N-labeled carbamoyl-PROXYL and carboxy-PROXYL, but this effect is significantly smaller in the high-viscosity sample (85% glycerol) of ^{14}N-labeled TEMPO. The line width broadening increases by ~50% in the high-viscosity samples (85% glycerol) of ^{14}N-labeled TEMPONE radical. The correlation time increases (~30 times) for 2 mM ^{14}N-labeled carbamoyl-PROXYL and carboxy-PROXYL solution in 85% glycerol. There is no appreciable change in the correlation time for 2 mM ^{14}N-labeled TEMPO solution in 85% glycerol. The rotational correlation time increases (~13 times) for 2 mM ^{14}N-labeled TEMPONE in the high-viscosity liquid mixture (pure water/glycerol). This ESR behavior implies that TEMPONE is a suitable spin probe for in vivo/in vitro studies in high-viscosity biological fluids, which has the narrowest line width and also acts as a viscosity-prone spin probe. The DNP parameters are correlated with the ESR parameters. These results will be useful for the development of ESRI/OMRI modalities in high-viscosity fluids for in vivo/in vitro imaging and also help in optimizing the ESR/OMR parameters in ESRI/OMRI techniques.

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