CHAPTER-IV

PERMEABILITY STUDIES OF REDOX-SENSITIVE NITROXYL SPIN PROBES THROUGH LIPID MEMBRANES USING AN L-BAND ESR SPECTROMETER

ABSTRACT

Electron spin resonance (ESR) studies were carried out for 2 mM ^{14}N-labeled deuterated 3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (MC-PROXYL) and 3-carboxy-2,2,5,5,-tetramethyl-pyrrolidin-1-oxyl (carboxy- PROXYL) in pure water and various concentrations of liposomal solution by using an L-band ESR spectrometer. The ESR parameters such as line width, hyperfine coupling constant, g-factor, rotational correlation time and partition parameter were reported for the samples. The changes in the line width were observed for ^{14}N-labeled deuterated MC-PROXYL and carboxy-PROXYL in liposomal solution. The hyperfine coupling constant was observed for both nitroxyl spin probes. The permeable and impermeable nature of nitroxyl radicals were demonstrated using the ESR L-band spectra. The rotational correlation time increases with increasing concentration of liposome. The partition parameter for ^{14}N-labeled deuterated MC-PROXYL in liposomal solution increases with increasing concentration of liposome, which reveals that the nitroxyl spin probe permeates into lipid membrane. The lipid peaks were observed for 2 mM ^{14}N-labeled deuterated MC-PROXYL in 200, 300 and 400 mM liposomal concentrations. The lipid peaks were not observed for ^{14}N-labeled deuterated carboxy-PROXYL in liposomal solution. These results indicate the permeable and impermeable nature of ^{14}N-labeled deuterated nitroxyl spin probe.

4.1 INTRODUCTION

Liposomes are spherical, colloidal particles with self-closed structures formed by one or several concentric lipid bilayers with an aqueous phase inside and between the layers. They are biocompatible, biodegradable, pH-sensitive and non-immunogenic in nature, which makes them ideal drug carrier systems in therapeutics. The hydrophilic and hydrophobic pharmaceuticals can entrap in the internal water compartment and membrane of the liposomes [1–3]. The liposomes of various compositions can extensively bind to cell surfaces, the size of liposomes and the types of cells are fundamental for an efficient capture by cells. Recently, liposomes have been used to entrap drugs for ultrasound-controlled drug release, ultrasound enhanced drug delivery and gene delivery systems [4,5]. The structural, thermodynamic and kinetic parameters of lipids for distinct applications are based on properties such as size, colloidal behaviour and phase transitions [6].

Electron spin resonance (ESR) is a reproducible technique, which presents the advantage of exploring the membrane bilayer in a non-invasive manner [7], providing important data concerning molecular organization in cell membranes that should help assess the actual condition and functionality of the relevant liposomes [8]. ESR is also used to investigate the dynamics of nitroxyl radicals in phosphatidylcholine based lipid membranes [9–11]. Paramagnetic nitroxides have been used as spin probes for in vivo ESR spectroscopic technique used in clinical diagnosis [12]. Recently, in vivo ESR spectroscopy and imaging have been widely used to investigate free radical distribution and metabolism in tissues, organs, and whole body of small animals. In vivo ESR spectroscopy performed at low microwave frequencies (L-band and low frequencies) allows in vivo measurement of nitroxyl radicals administered to experimental animals. This technique has been used to non-

invasively measure generation of free radicals/reactive oxygen species (ROS) in various animal disease models [13]. In order to understand the ESR parameters at low microwave frequency, the present work was carried out using an L-band ESR spectrometer. ESR and Overhauser-enhanced magnetic resonance (OMR) imaging studies were carried out using the high spin concentration due to its low sensitivity at low microwave frequency (~1 GHz). Murali et al. and Utsumi et al. [14–16] reported ESR/OMR imaging and phantom studies using the spin probe concentration from 1 to 2 mM.

The time-resolved ESR studies of spin-labeled lipid in membranes show the details of lipid chain librational dynamics and the penetration of water into membranes [17]. Recently, Mirosavljevic et al. [18] reported that the interfacial properties of egg-PC liposomes as a function of cholesterol concentration, long chain lipid label and TEMPO-stearate were used. The nitroxide moiety of these spin labels is located within head groups region of phospholipids. Above 29 mol% of cholesterol, TEMPO-stearate reported two different nitroxide environments of similar dynamic properties, differing mainly in the amount of water bound in the near neighbourhood of the nitroxide moiety. The presence of two different environments of TEMPO-stearate above 29 mol% of cholesterol differing mainly by the amount of water bound in the near neighborhood of the N–O bond may be due to the formation of cholesterol enriched and cholesterol poor domains in accordance with results reported [19,20]. The antioxidant effects of water and lipid soluble nitroxyl radicals in liposomes directly depend on both nitroxide concentration and lipophilicity [21]. Researchers used the ESR technique and observed the interaction of lipids in liposomes with ovalbumin [22], chlorpromazine [23] and tetraphenyl-porphyrin derivatives [24] to depend on the physical state of the membrane, pH, temperature and cholesterol control. ESR imaging (ESRI) and OMR imaging (OMRI) techniques are powerful biomedical tools for in vivo free radical imaging. These techniques are used to monitor the biological

processes and ROS generation at cellular and subcellular levels [15, 16, 25-28].

The nitroxyl radicals can undergo one-electron oxidation or reduction in the presence of appropriate reactants and are sensitive to reduction and oxidation processes. Recently, Utsumi et al. [15] demonstrated the simultaneous molecular imaging of reduction and oxidation processes monitored by OMRI with ^{14}N- and ^{15}N-labeled nitroxyl radicals. Recently, Utsumi et al. [29] reported the dynamic nuclear polarization of redox-sensitive nitroxyl spin probes in liposomal solution. The ESR parameters reveal the physical and chemical properties of free radicals distributed into the biological systems. In order to study the permeable and impermeable nature of nitroxyl spin probes in liposomal solution and optimize the liposome concentration for phantom studies, the ESR studies on 2 mM concentration of ^{14}N-labeled deuterated MC-PROXYL and carboxy-PROXYL in liposomal solution as a function of liposome concentration using an L-band ESR spectrometer is reported.

4.2 MATERIALS AND METHODS

The spin probe ^{14}N-labeled deuterated 3-carboxy-2,2,5,5,-tetramethyl-pyrrolidin-1- oxyl (carboxy-PROXYL) and L-a Phosphatidylcholine (Egg PC) were purchased from Aldrich Chemical Co, St. Louis, MO, USA. The ^{14}N-labeled deuterated 3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (MC-PROXYL) was synthesized as described earlier [30]. All other chemicals were analytical grade.

4.2.1 Multilamellar Liposome Preparation

Multilamellar liposomes were prepared as follows [28]: Egg phosphatidylcholine (Egg PC) was first dissolved in chloroform which was

later removed by rotary evaporation, yielding a thin film on the sides of a round bottom flask. The film was thoroughly dried under vacuum for about 2 h. The dry lipids were suspended in nitroxyl spin probe containing phosphate buffer solution (PB), pH 7.4 and by vortex agitation liposome dispersions with final lipid concentration of 100, 200, 300 and 400 mM were prepared and briefly sonicated.

4.2.2 Particle Size Determination

The particle size of the liposome was measured by a Zetasizer Nano light-scattering spectrophotometer (Malvern Instruments, Malvern, England). The size of the liposome was determined as ~100 nm, after extrusion using polycarbonate filter.

4.2.3 ESR Measurements

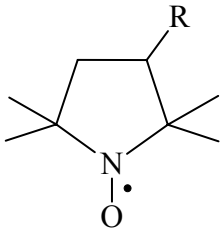
This study was carried out with an L-band ESR spectrometer (JEOL, Tokyo). The ESR spectra were recorded for 2 mM ^{14}N-labeled deuterated permeable MC-PROXYL in pure water, 100, 200, 300, 400 mM concentrations of liposomal solution and impermeable carboxy-PROXYL in pure water, 400 mM concentration of liposomal solution using L-band ESR technique by varying the magnetic field, 33.3–38.3 mT. The ESR acquisition parameters were as follows: modulation frequency, 100 kHz; field modulation amplitude, 0.1 mT; radio frequency power, 10 mW; sweep width, ±2.5 mT and radio frequency, 1.032 GHz. The measured efficiency of the resonator is 77 $\mu T/W^{1/2}$. The ESR spectrum was recorded in the first-derivative mode at 27^{o}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 and loaded in a 2 cm diameter ESR phantom. For each measurement, the phantom was filled with sample for a length of 5 cm and volume 10 ml. To prevent water loss during the measurements, the phantoms

were sealed at both sides. Temperature was controlled using a controller with water as a coolant.

4.3 RESULTS AND DISCUSSION

The ESR spectra of 2 mM concentration of ^{14}N-labeled deuterated permeable MC- PROXYL in pure water, 100, 200, 300, 400 mM concentration of liposomal solution and impermeable carboxy-PPROXYL in pure water, 400 mM concentration of liposomal solution are shown in the Figs. 4.1 and 4.2. Table 4.1 shows the structures, abbreviations and n-octanol/water partition coefficients ($P_{O/W}$) of nitroxyl radicals [28]. The ESR parameters such as line width, hyperfine coupling constant, g-factor, rotational correlation time and partition parameter were observed and given in the Table 4.2.

Table 4.1 Structures, abbreviations and n-octanol/water partition coefficients ($P_{O/W}$) of nitroxyl radicals

Ring structure	Substituent (R)	Abbreviation	Po/w
	-COOH -$COOCH_3$	Carboxy-PROXYL Methoxy carbonyl-PROXYL	0.02 ± 0 9.50 ± 0.11

4.3.1 Line Width

The ESR line width values are given in Table 4.2. The line width of ^{14}N-labeled deuterated permeable MC-PROXYL and impermeable carboxy-PROXYL in pure water were obtained as 79 and 75 μT, respectively. The increase in line width of nitroxyl spin probe was observed as ~50 % in liposomal solution. The line broadening of nitroxyl spin probe in the liposomal solution, is likely because dipolar and spin-exchange interactions can

broaden the ESR resonance. The line broadening indicates the viscous nature of the liposomal solution. In high viscous medium, the nitroxyl spin probes have less mobility, which also leads to the line broadening mechanism. These results agree well with the previous study [31].

Table 4.2 ESR parameters of 2 mM ^{14}N-labeled deuterated permeable MC-PROXYL in pure water, 100, 200, 300, 400 mM concentration of liposomal solution and impermeable carboxy-PROXYL in pure water, 400 mM concentration of liposomal solution

Samples		Central line width (μT)	Hyperfine coupling constant A_{iso} (mT)		g-factor	Rotational correlation time τ (s) x10^{-11}
			Water peak	Lipid peak		
MC-PROXYL	Pure water	79	1.438	-	2.0594	1.55
	100 mM liposome	116	1.434	-	2.0618	10.04
	200 mM liposome	116	1.434	1.275	2.0620	19.42
	300 mM liposome	116	1.434	1.282	2.0620	-
	400 mM liposome	116	1.433	1.289	2.0620	-
carboxy-PROXYL	Pure water	75	1.457	-	2.0601	1.58
	400 mM liposome	116	1.454	-	2.0623	2.64

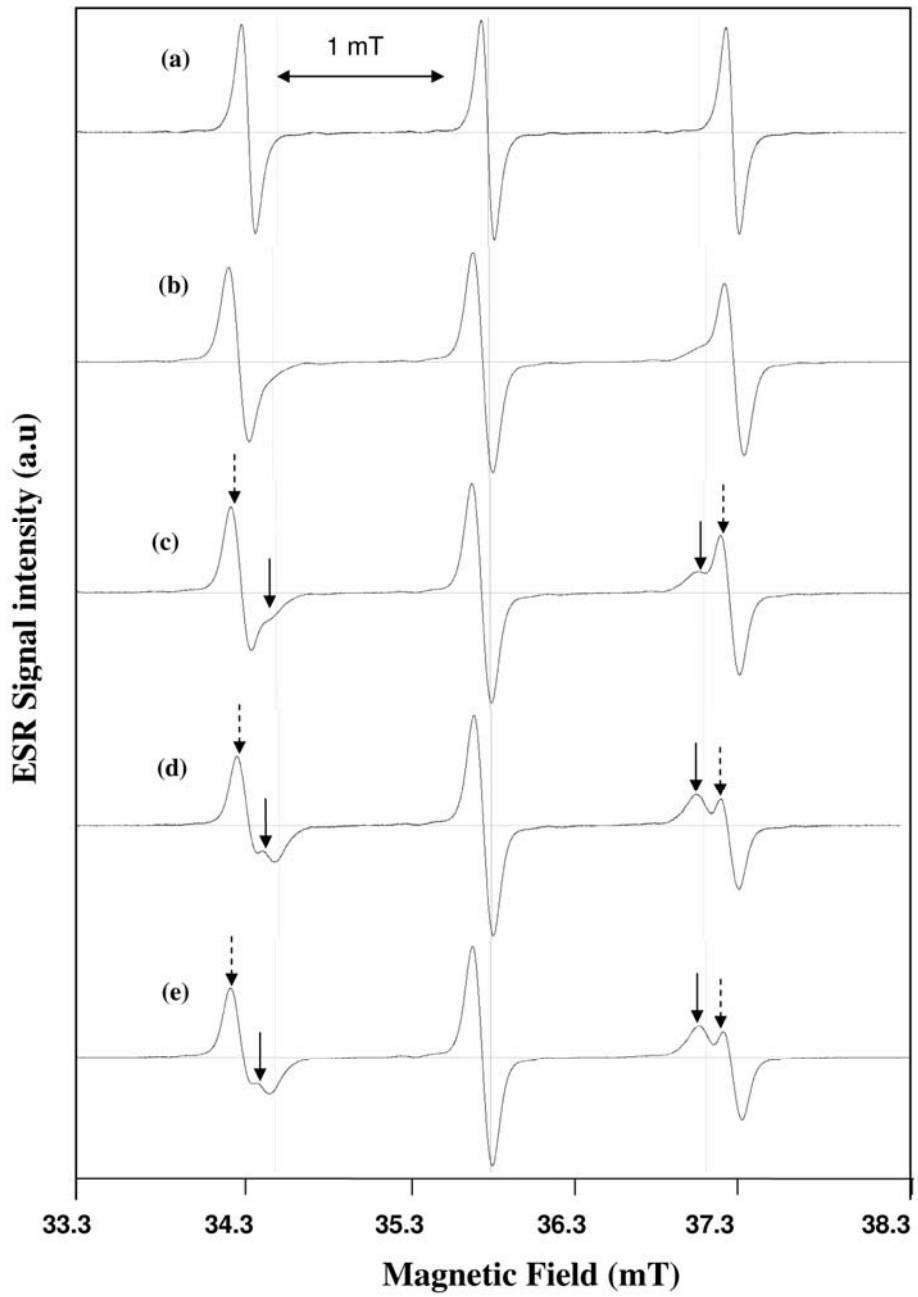


Fig. 4.1 ESR spectra of 2 mM ^{14}N-labeled deuterated permeable MC-PROXYL in **(a)** pure water, **(b)** 100 mM, **(c)** 200 mM, (**d)** 300 mM and **(e)** 400 mM concentrations of liposomal solution (the dotted line arrow indicates the aqueous peak and solid line arrow indicates the lipid peak).

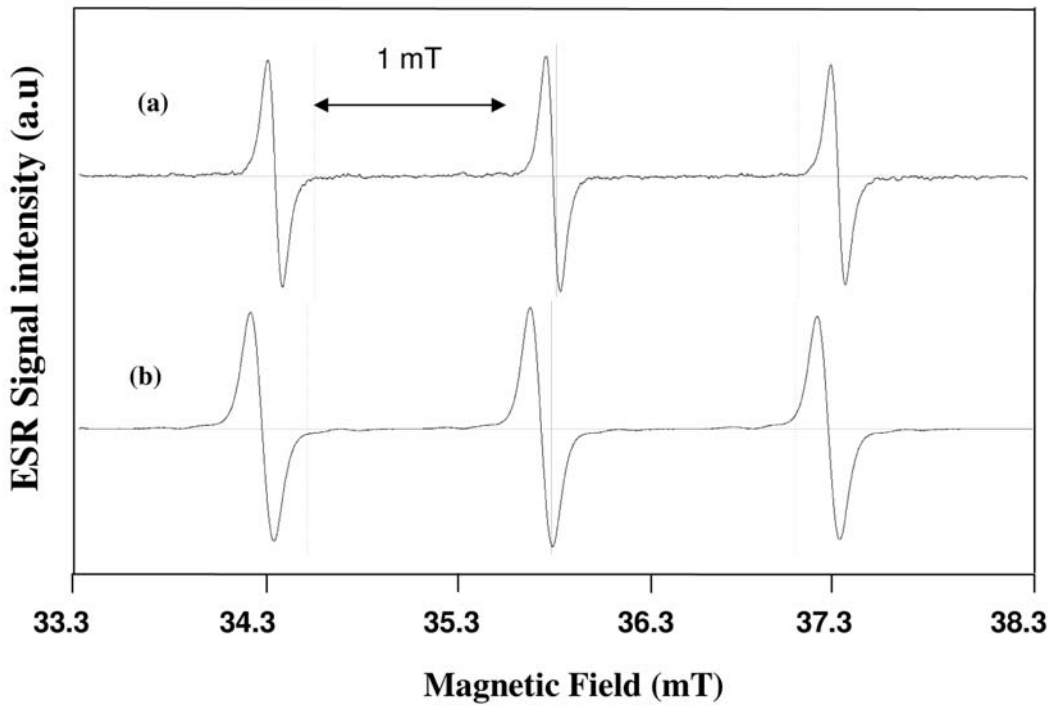


Fig. 4.2 ESR spectra of 2 mM ^{14}N-labeled deuterated impermeable carboxy-PROXYL in **(a)** pure water and **(b)** 400 mM concentration of liposomal solution.

4.3.2 Hyperfine Coupling Constant and g-Factor

The deuterated nitroxyl spin probes have a small line width due to the lower magnetic moment of deuterium. Therefore, the deuterated ^{14}N-labeled MC- PROXYL and carboxy-PROXYL have been used. The ESR low and high field lines were well resolved for ^{14}N-labeled deuterated permeable MC-PROXYL in 200, 300 and 400 mM concentration of liposomal solution; one corresponds to the aqueous peak and another one corresponds to the lipid peak, which are shown in Fig. 4.1c–e. The hyperfine coupling constant was obtained as 1.44 mT, which corresponds to the aqueous peak. The hyperfine coupling constant value 1.29 mT corresponds to the

lipid peak, which is less than that of the aqueous peak. This ESR behaviour can be explained in terms of the fermi contact interaction in lipid phase and aqueous phase. The g-factor values were estimated for both nitroxyl spin in pure water and liposomal solution. These values are listed in Table 4.2.

4.3.3 Rotational Correlation Time

Cruz et al. [32] reported the rotational dynamics of spin-labeled surfactant associated proteins SP-B and SP-C in dipalmitoyl-phosphatidylcholine and dipalmitoylphosphatidyl glycerol bilayers. The rotational correlation time τ_R 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 τ_R is given by an empirical formula [33,34].

$$\tau_R = 6.5 \times 10^{-10} \, \Delta B_0 [(h_0 / h_{-1})^{1/2} - 1] \qquad (4.1)$$

where h_{-1} and h_0 are the heights 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.

The rotational correlation time for 2 mM ^{14}N-labeled deuterated permeable MC-PROXYL in pure water, 100, 200 mM concentration of liposomal solution and impermeable carboxy-PROXYL in pure water, 400 mM, concentration of liposomal solution, which are shown in Table 4.2. The rotational correlation time increases with increasing concentration of liposome for ^{14}N-labeled deuterated permeable MC-PROXYL. This dynamic behaviour is due to the permeable nature of nitroxyl spin probe. The increased rotational correlation time indicates the less mobile nature of the spin probe in liposomal solution. The change in rotational correlation time

observed for impermeable ^{14}N-labeled deuterated carboxy-PROXYL is not so significant compared with permeable ^{14}N- labeled deuterated permeable MC-PROXYL.

Table 4.3. The partition parameter and permeability of ^{14}N-labeled deuterated permeable MC-PROXYL in 200, 300 and 400 mM concentration of liposomal solution

Samples		Partition parameter f	Permeability R
MC-PROXYL	200 mM liposome	0.208	3.798
	300 mM liposome	0.329	2.044
	400 mM liposome	0.344	2.020

4.3.4 Membrane Permeability and Partition Parameter

Membrane permeability is defined as the capacity of solute leakage out of cells or penetrating into cells not through active transport [35]. The R value, defined as the ratio of height of the aqueous (W) to lipid (L) component in the high-field region of the ESR spectrum, which is used to quantify the membrane permeability in different tissues [36]. The nitroxyl spin probes can be used to measure membrane permeability. The R values were calculated for 2 mM ^{14}N-labeled deuterated permeable MC-PROXYL in 200, 300 and 400 mM concentrations of liposomal solution and shown in Table 4.3. The R value decreases with increasing concentration of liposome. The decrease in R value describes an increase of membrane permeability. The partition between the lipid and aqueous phase can be directly obtained by assuming the

relative amplitudes of the number of spin label molecules in hydrophobic H and polar P environments [37].

The partition parameter, f can be expressed as:

$$f = \frac{H}{H + P} \tag{4.2}$$

The partition parameters were observed for deuterated permeable MC-PROXYL in 200, 300 and 400 mM concentration of liposomal solution. The estimated partition parameter from the spectra increases with increasing concentration of liposomal solution. The partition parameter value changes only in the range of 200 and 300 mM concentration of liposome, but no significant change was observed for 400 mM concentration. Therefore, the liposome concentration was optimised as 300 mM for phantom studies. The permeable nitroxyl spin probe MC-PROXYL with high partition co-efficient value (9.5) permeates into liposomal solution, but the impermeable nitroxyl spin probe carboxy-PROXYL with low partition co-efficient value (0.02) does not permeate into lipid membrane. This phenomenon can be illustrated from Figs. 4.1 c–e and 4.2b, respectively. The partition parameter values of nitroxyl spin probes agree well with the permeable and impermeable nature of nitroxyl spin probes.

4.4 CONCLUSIONS

The permeable and impermeable nature of ^{14}N-labeled deuterated MC-PROXYL and carboxy-PROXYL was demonstrated using an L-band spectrometer. The liposome concentration was optimised as 300 mM for phantom studies. The linewidth broadening was observed for nitroxyl spin probe in liposomal solution. The hyperfine coupling constant corresponding to aqueous and lipid peak was estimated. The rotational correlation time of

nitroxyl spin probe in the liposomal solution indicates the less mobile nature in high viscous medium. The partition parameter reveals the permeable and impermeable nature of nitroxyl spin probe. The membrane permeability (R value) decreases with increasing concentration of liposome. The decrease in R value describes an increase of membrane permeability. This study illustrates that the ESR technique can be used to differentiate between the intra- and extra-membrane water by loading the liposomes vesicles with a lipid permeable nitroxyl spin probe.

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