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# Diffusion Studies of Redox-Sensitive Nitroxyl Spin Probes through Bilayer Lipid Membranes using 300 MHz Electron Spin Resonance Spectrometer

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**Abstract:** Electron spin resonance (ESR) studies were carried out for 2mM $^{14}$N-labeled deutrated 3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (MC-PROXYL) and 3–carboxy-2,2,5,5,-tetramethyl-1-pyrrolidinyloxy (carboxy-PROXYL) in pure water and various concentrations of liposomal solutions by using 300 MHz ESR spectrometer. The ESR parameters such as the line width, hyperfine coupling constant, rotational correlation time, g-factor, partition parameter and permeability were reported for the samples. The line width broadening was observed for 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 spin probes was demonstrated. The rotational correlation time increases with increasing concentration of liposome. The partition parameter increases with increasing concentration of liposome for MC-PROXYL, which indicates that the nitroxyl spin probes diffuse into lipid membrane. The permeability (R) value decreases with increasing concentration of liposome, which reveals that the increase of membrane permeability. The peaks correspond to lipid phase were observed for MC-PROXYL in liposomal solution, but that peak was not resolved for carboxy-PROXYL. These results confirm the permeable and the impermeable nature of nitroxyl spin probes.

**Keywords:** Electron spin resonance, Free radicals, Liposomes, Permeability.

## 1. Introduction

Biological membranes are dynamic structures with a lipid composition known to be very asymmetric and heterogenous [1]. Liposomes are self-closed structures composed of amphiphilic lipids that form a bilayer around an aqueous compartment and have been extensively used as cell membrane models. Liposomes present a membrane structure similar to the cellular one, in which the lipophilic hydrocarbon region is sandwiched between two ordered polar head group regions [2]. Liposomes are suitable models of cell membrane to study the influence of several parameters like viscosity or surface charge density on the distribution properties of hydrophobic sensitizer in normal and tumor tissues [3].

Electron spin resonance spectroscopy of spin labelled lipids has long proved to be a valuable method for studying rotational dynamics and molecular ordering in lipid bilayers and biological membranes through the detection of unpaired electrons and radicals in biological molecules [4]. Electron spin resonance spectroscopy is a powerful non-invasive spectroscopic tool that can be used to monitor drug release processes in vitro and in vivo [5].

The magnetic interactions of nitroxide spin labels are extremely sensitive to motion on the nanosecond time scale in the ESR X-band frequency (~9 GHz), which is significant for the dynamics of biomolecules [4]. The X-band electron paramagnetic resonance (EPR) is unsuitable for the study of

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intact animals, or even excised organs. For EPR measurements on these types of samples, it is necessary to reduce the frequency of electromagnetic radiation used, so that it will not be completely adsorbed by non-resonant interactions. This is done by reducing the magnetic fields at which the spectrometer operates. Recently, Research groups have developed EPR instrumentation with frequency range between 200 MHz to 1.5 GHz. ESR spectrometer with 300 MHz is used to detect free radicals injected or implanted into living animals. Even at these frequency ranges there remains some degree of adsorption in biological samples, limiting the penetration depth of electromagnetic radiation. As a result, L-band spectrometers tend to be used for studies of animals no larger than mice (~ 25 g body weight), while rats (~250 g body weight) can be examined by ESR spectrometers operating in the radio frequency [6-8].

The transport of many molecular species such as glucose, amino acids, metal ions of biological interest invokes carriers and channel proteins, passive diffusion within biomembranes is the predominant mechanism for many permeants, including lipophilic drug molecules as well as low molecular weight polar compounds. These studies have provided insight into the mechanism by which the chemical composition of bilayer lipids and physical states of the membranes [9]. Using lipid spin labels with EPR monitoring groups (free radical nitroxide moieties) located at different depths in the membrane, profiles of different membrane properties across the bilayer can be obtained [10]. The spin label EPR approach can provide precise evaluations of molecular dynamics and ordering of stratum corneum lipids, which also may be used to investigate drug-lipid interaction directly in native stratum corneum as well as different cases of skin diseases and lesions [11]. Spin labeling ESR studies are helpful to understand the properties of oxidized phospholipid-containing lipid vesicles [12]. The interaction of antitumoral drug daunorubicin with liposomes shows that the electrostatic and hydrophobic forces are necessary for the stabilization of antracyclines in the lipid bilayer [13].

The dynamic nuclear polarization studies of redox-sensitive membrane permeable and impermeable nitroxyl spin probes in liposomal solution was reported. This study illustrates that Overhauser Magnetic Resonance Imaging (OMRI) can be used to differentiate between the intra and extra membrane water by loading the liposome vesicles with a lipid permeable nitroxyl spin probe [14]. Benial et al. reported that the permeability studies of redox sensitive nitroxyl spin probes through lipid membranes using an L-band ESR spectrometer [15]. Many researchers recently reported that the ESR and OMR invivo experiments in small animal at the low frequency (300 MHz). In order to understand the ESR parameters, here we report the diffusion process of permeable and impermeable nitroxyl radicals in pure water and various concentrations of liposomal solutions by using 300 MHz ESR spectrometer.

## 2. Materials and Methods

### 2.1. Chemicals

The spin probe, $^{14}$N-labeled deuterated 3-carboxy-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (carboxy-PROXYL), L-α Phosphatidylcholine (Egg PC) were purchased from Aldrich Chemical Co, St. Louis, MO, USA.The $^{14}$N-labeled deutrated 3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (MC-PROXYL) was synthesized as described earlier [16]. All other chemicals were analytical grade.

### 2.2. Preparation of multilamellar liposomes

Multilamellar liposomes were prepared as follows [16]: 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 two hours. The dry lipids were suspended in spin probe containing phosphate buffer solution (PB), pH = 7.4 and by vortex agitation, liposome dispersions with final lipid concentration 100, 200, 300 and 400 mM were prepared. Nitroxyl spin probes were added to the liposomal solution and briefly sonicated for one hour at 23ºC.

### 2.3. Particle size determination

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

### 2.4. ESR measurements

The work described in this report was carried out by 300 MHz ESR spectrometer at 23ºC [16]. The ESR spectra were recorded for 2 mM $^{14}$N-labeled deuterated permeable MC-PROXYL in pure water, 100, 200, 300, 400 mM concentration of liposomal solutions and impermeable carboxy- PROXYL in pure water, 400 mM concentration of liposomal solutions by varying the magnetic field 8-13 mT with modulation frequency 100 kHz, field modulation amplitude 0.1 mT, time constant 30 ms, radio frequency power 100 mW, sweep width ±2.5 mT and radio frequency 300 MHz. The ESR spectrum was recorded in the first derivative mode at 27°C. In order to remove oxygen from the solution, argon gas was passed over the samples about two hours. The samples were prepared using the phosphate buffer solution at pH 7.4. The samples with different concentrations of liposomal solution contents were loaded into a 2 cm diameter ESR phantom. For each measurement, the phantom was filled with samples for a length of 5 cm and volume 10 ml. To avoid water loss during the measurements the phantoms were sealed on both sides. The temperature was controlled by using a controller with water as the coolant.

## 3. Results and Discussion

In the heterogeneous membrane, the regions have one or more different fluidity properties and it is called as domains. The experimental ESR spectrum is a superposition of spectral components that correspond to the spin probes in the membrane domain types with different fluidity characteristics. The ESR spectrum for 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 were shown in Figs. 1 and 2. The ESR parameters such as the line width, hyperfine coupling constant, rotational correlation time and g-factor were listed in Table 1. Table 2 shows the partition parameter, permeability and order parameter of MC-PROXYL in 300 and 400 mM concentration of liposomal solution.

### 3.1. Line width

The line width of the ESR spectra visualizes the ordering and dynamics of the spin probe, which also reflect the motional characteristics of its surroundings. The observed ESR central line width values for 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 were listed in Table1. The line width of deuterated permeable MC-PROXYL in pure water and 400 mM concentration of liposomal solution were estimated as 79 and 110 μT respectively. The line width of impermeable carboxy-PROXYL in pure water and 400 mM concentration of liposomal solution were estimated as 78 and 112 μT respectively. The line width value was observed as ~40% higher for both nitroxyl radicals in 400 mM concentration of liposomal solution. The viscous nature of the liposomal solution leads to the line broadening mechanism in the ESR spectrum, which also indicates the less mobility of the nitroxyl spin probe in liposomal solution [18].

**Table 1.** ESR parameters of $^{14}$N-labeled deuterated permeable MC-PROXYL in pure water, 100, 200, 300 and 400 mM concentration of liposomal solution and impermeable carboxy- PROXYL in pure water and 400 mM concentration of liposomal solution.

| Sample | Central line width ΔB (μT) | Hyperfine coupling constant $A_{iso}$(mT) | | Rotational correlation time $\tau_R$ (s) ($x10^{-11}$) |
|---|---|---|---|---|
| | | Water peak | Lipid peak | |
| MC-PROXYL | | | | |
| Pure water | 79 | 1.430 | - | 1.35 |
| 100 mM liposome | 108 | 1.425 | - | 7.82 |
| 200 mM liposome | 108 | 1.421 | - | 12.26 |
| 300 mM liposome | 110 | 1.421 | 1.271 | - |
| 400 mM liposome | 110 | 1.421 | 1.282 | - |
| carboxy-PROXYL | | | | |
| Pure water | 78 | 1.429 | - | 1.31 |
| 400 mM liposome | 112 | 1.420 | - | 3.08 |

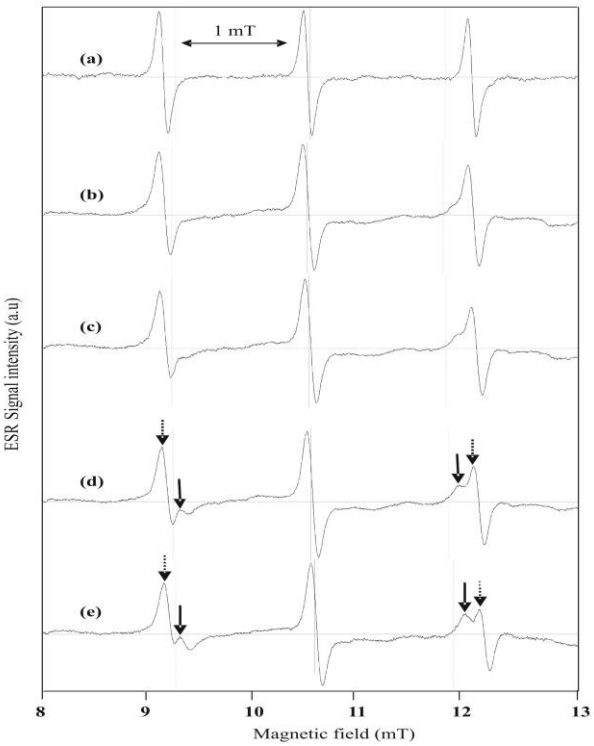


**Figure 1.** ESR spectra of 2 mM $^{14}$N-labeled deuterated permeable MC- PROXYL in a) pure water b)100 c) 200 d) 300 and e) 400 mM concentration of liposomal solution. The solid line arrow indicates the lipidic component and the dotted line arrow indicates the aqueous component

### 3.2 Hyperfine coupling constant and g-Factor

The Hyperfine coupling constant and g-factor values were observed and listed in Table 1. The ESR low field and high field lines corresponds to aqueous peak and lipid peak were resolved for permeable MC-PROXYL in 300 and 400 mM concentrations of liposomal solutions, which are shown in Figs. 1d and 1e. The hyperfine coupling constant value was observed as ~1.430 mT for permeable MC-PROXYL and impermeable carboxy- PROXYL in pure water. The hyperfine coupling constant value for permeable MC-PROXYL in 400 mM concentration of liposomal solution were estimated as 1.421 and 1.282 mT, which correspond to aqueous peak and lipid peak respectively. This ESR behavior can be explained in terms of the Fermi contact interaction. The lipid peak was not observed for impermeable carboxy-PROXYL in 400 mM concentration of liposomal solution. The g-factor value was observed for both nitroxyl spin probe in pure water and liposomal solution. The observed g-value (~2.0340) indicates the isotropic nature of the solvent.

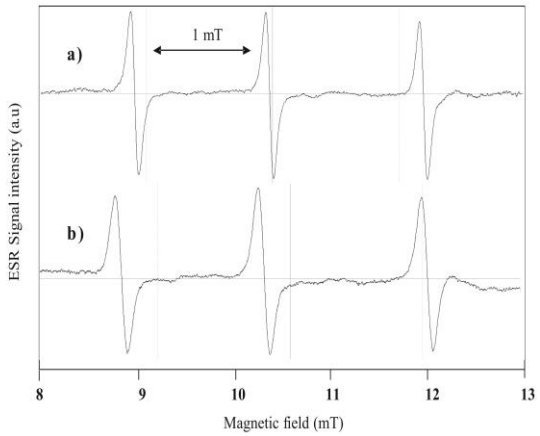


**Figure 2.** ESR spectra of 2 mM $^{14}$N-labeled deuterated impermeable carboxy-PROXYL in a) pure water and b) 400 mM concentration of liposomal solution.

### 3.3. 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 rate of rotation or tumbling of the spin label influences the lineshape of the ESR spectrum. The rotational correlation time $\tau_R$ is a parameter to express the mobility of spin probes in their environment. The $\tau_R$ can be obtained from the ESR spectral line width and relative intensities. The rotational correlation time is given by an empirical formula [19,20]

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

where $h_{-1}$ and $h_0$ are the heights of the high-field and central lines in the ESR spectra, respectively, and $\Delta B_0$ is the line width of the central line in gauss. The rotational motion of the spin probe was assumed to be isotropic. This expression is valid only for fast isotropic motion in solution and can be used in the membrane only to compare relative differences in the spin probe dynamics between different cell lines and different treatments [21]. The empirical correlation time is inversely proportional to membrane fluidity. The membrane fluidity is also described with motional characteristics of the membrane phospholipid alkyl chains and is inversely proportional to average membrane viscosity [22].

The rotational correlation time for permeable MC-PROXYL in pure water, 100, 200 mM and impermeable carboxy-PROXYL in pure water, 400 mM concentration of liposomal solution are listed in Table 2. The rotational correlation time increases with increasing concentration of liposome for permeable MC-PROXYL. The rotational correlation time for MC-PROXYL in pure water and 200 mM concentration of liposomal solution were estimated as 1.35 and 12.26 x $10^{-11}$ s respectively. The increased rotational correlation time is mainly due to the viscous nature of the sample and the dynamic behaviour of the permeable MC-PROXYL. This ESR behaviour indicates the less mobility of the nitroxyl spin probe in liposomal solution. The rotational correlation time observed for impermeable carboxy-PROXYL is not so significant compared with permeable MC-PROXYL.

**Table 2.** The Partition parameter and Permeability of $^{14}$N-labeled deuterated permeable MC-PROXYL in 300 and 400 mM concentration of liposomal solution

| Sample | Partition parameter $f$ | Permeability R |
|---|---|---|
| MC-PROXYL | | |
| 300 mM liposome | 0.279 | 2.578 |
| 400 mM liposome | 0.340 | 1.934 |

### 3.4. Membrane permeability

Membrane permeability (R) was measured by the ESR method using the spin probe in the membrane phospholipid bilayer. Membrane permeability is defined as the capacity of solute leakage out of cells or diffusing into cells not through active transport [23]. The R value, defined as the ratio of aqueous (W) to lipid (L) component in height in the high-field region of the ESR spectrum, which is used to quantify the membrane permeability in different tissues [24]. The nitroxyl spin probes can be used to measure membrane permeability. The R values were calculated for permeable MC-PROXYL in 300 and 400 mM concentration of liposomal solution and shown in Table 2. The R value decreases with increasing concentration of liposome, which reveals that the increase in membrane permeability.

### 3.5. Partition parameter

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 [25].

The partition parameter $f$ can be calculated by using the formula:

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

The partition parameters were observed for permeable MC-PROXYL in 300 and 400 mM concentration of liposomal solution. The estimated partition parameter increases with increasing concentration of liposomal solution. The partition parameter value is 25% higher for permeable MC-PROXYL in 400 mM concentration of liposomal solution compared with 300 mM concentration of liposomal solution. Therefore, the liposome concentration was optimised as 400 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 [17]. The permeable and impermeable nature can be illustrated from Figs. 1c–1e and 2b respectively.

## 4. Conclusion

The permeable and impermeable nature of MC-PROXYL and carboxy-PROXYL was demonstrated by using low frequency (300 MHz) ESR spectrometer. The liposome concentration was optimized as 400 mM for phantom

studies. The line width 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 nitroxyl spin probe increases with increasing concentration of liposomal solution, which 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 for MC-PROXYL, which reveals the increasing nature of membrane permeability. This study illustrates that the ESR technique at low frequency can be used to differentiate between the intra- and extra-bilayer lipid membranes water by loading the liposomes vesicles with a lipid permeable nitroxyl spin probe.

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