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Proxyl Nitroxide of Lithocholic Acid: A Potential Spin Probe for Model Membranes

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Abstract—A new steroidal proxyl (2,2,5,5-tetramethylpyrrolidine-*N*-oxyl) nitroxide (SPN), with the proxyl nitroxide moiety in the pendant side chain of the steroid, has been synthesized. Its localization in lipid bilayers was ascertained with the help of ^{1}H NMR and ^{31}P NMR experiments. The effects of the nitroxide group in SPN incorporated into the bilayer on ^{13}C relaxation times are interpreted qualitatively in terms of localization of the nitroxide group within the bilayer structure. The nitroxide SPN was used to monitor changes in membrane fluidity and permeability induced by local anaesthetics, mepivacaine and xylocaine and the antikeratinizing agent, azelaic acid. The results conclusively proved the applicability of the new steroidal proxyl nitroxide (SPN) as a potential spin probe for spin labeling studies.

Introduction

Steroidal nitroxides in which the nitroxide moiety is rigidly attached to the main steroidal skeleton[1–3] have been extensively used[4–7] for studying changes in the structure and functions of biomembranes. The rigidly attached doxyl (4,4-dimethyloxazolidine-*N*-oxyl) nitroxides have largely been used[4] in spin labeling studies of oriented multibilayers of lipids. Owing to the rigid mode of fusion of the nitroxides to the steroidal skeleton, these nitroxides have not been used to monitor changes in microenvironmental fluidity which is characteristic of non-oriented model membranes such as liposomes. We have synthesized[8,9] doxyl, proxyl and tempo (2,2,6,6-tetramethylpiperidine-*N*-oxyl) nitroxides which have the nitroxide moieties in the side chain of the steroidal substrate lithocholic acid (3α-hydroxy-5β-cholanoic acid) **1**. These nitroxides are expected to have higher mobility owing to free rotation and are expected to exhibit greater versatility in spin labeling studies. Inherent differences in polarity between doxyl and proxyl nitroxides,[10] which could lead to differences in the mode of localization in biomembranes, prompted us to employ the newly synthesized proxyl nitroxide (SPN) **7**, as a spin probe for biomembranes. In this paper we report the localization of the nitroxide SPN in egg phosphatidyl choline (EPC) bilayers as ascertained with the help of ^{1}H NMR and ^{31}P NMR experiments. Supporting evidence in favour of the proposed mode of localization was obtained from ^{13}C spin-lattice relaxation time (T_1) measurements of the lipid in the presence of the nitroxide SPN. The nitroxide has been employed as an EPR sensitive probe to monitor the changes in fluidity and permeability of model membranes, made up of dipalmitoyl phosphatidyl choline (DPPC) and egg phosphatidyl choline (EPC) dispersions respectively, in the presence of local anaesthetics, mepivacaine[11] [*N*-(2,6-di-methyl phenyl)-1-methyl-2-piperidinecarboxamide], xylocaine[12] [2-(diethylamino)-*N*-(2,6-dimethylphenyl)-acetamide] and the antikeratinizing agent, azelaic acid[13] (1,7-heptanedicarboxylic acid).

Results and Discussions

Synthesis of SPN and its incorporation into lipid bilayers

The synthesis of the proxyl nitroxide (SPN) **7** has been achieved[9] (Scheme I) in an overall yield of 27% from lithocholic acid **1**. The key step in the synthetic sequence involved an esterification between a mixed anhydride **5**, derived from 3-carboxy proxyl **4** and the protected primary alcohol **3**. Cleavage of the tetrahydropyranyl ether group in the final step yielded the desired proxyl nitroxide (SPN) **7**.

The incorporation of SPN in liposomes has been ascertained by observing the EPR spectra of the nitroxide in rapidly tumbling solution state (Figure 1a) and that in

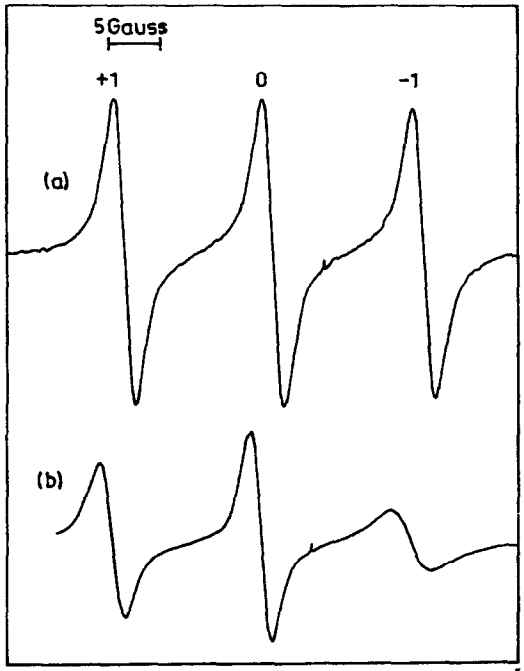
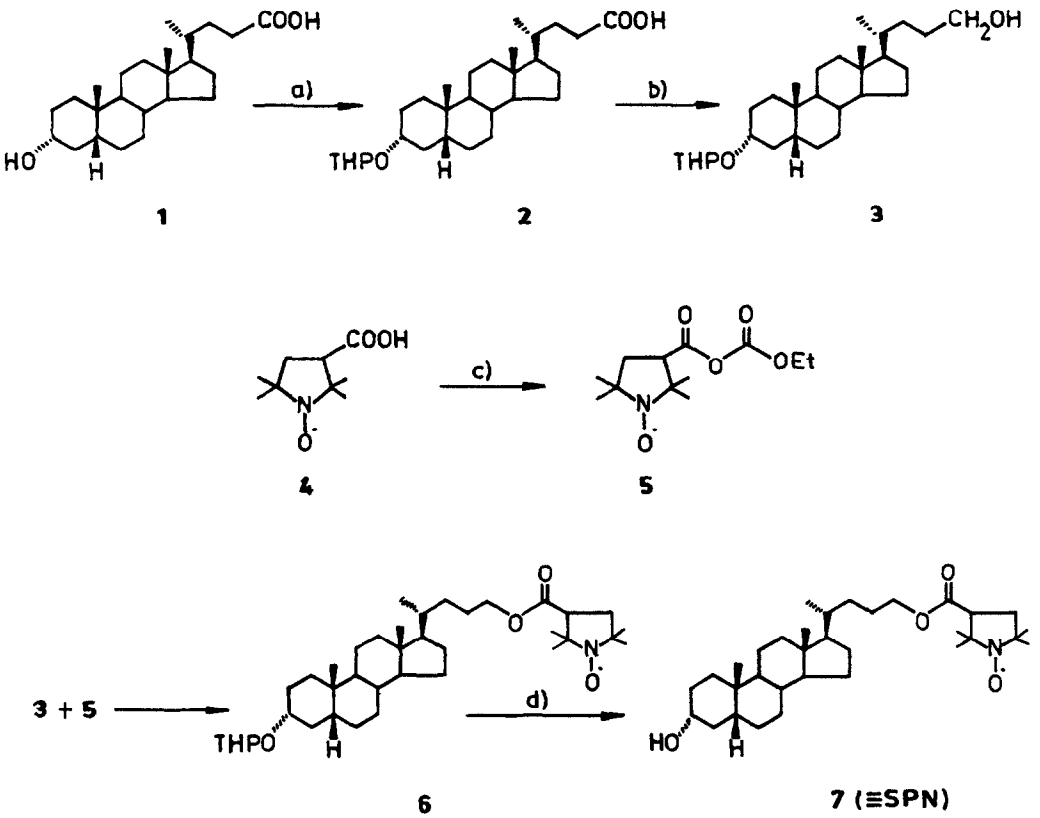


Figure 1. EPR Spectra of (a) spin label SPN in chloroform (10^{-5} M), (b) SPN incorporated into multilamellar vesicles of EPC. The concentrations of lipid and spin label are 100 mM and 1mM respectively



Conditions: (a) DHP, PTSA (catalytic amount), PhH–THF, r.t., 2 h; (b) LAH, THF, reflux, 3 h; (c) ethylchloroformate, triethylamine, THF, r.t., 48 h; (d) PTSA, CH_3OH, r.t., 0.5 h.

Scheme I. Synthesis of steroidal proxyl nitroxide 7 (SPN) from lithocholic acid 1.

the lipid matrix (Figure 1b). The hyperfine coupling constant of SPN in multilamellar vesicles is observed to be 14.40 G as against an isotropic value of 14.80 G obtained when it is in rapidly tumbling solution phase. An increase of 18.5% in the line width of the lowest field line in the EPR spectrum is observed. The differences observed in the line shapes of the highest and the lowest field lines in the EPR spectrum of the nitroxide in liposomes (Figure 1b) stems from the anisotropy of the nuclear hyperfine coupling tensor and g tensor of the nitroxide radicals in the lipid. The changes in the EPR line shapes, hyperfine coupling constant and the line width of the EPR spectra of SPN in liposomes suggest its incorporation in liposomes.

One notices that the EPR spectrum of SPN incorporated in liposomes (Figure 1b) exhibits a near isotropic behaviour. This can be attributed to two factors. Firstly, the proxyl nitroxide is in the flexible side chain of the steroid and hence exhibits higher mobility than that of the rigidly attached 3-doxyl substituted steroids. Secondly, it can also be explained with the help of the model proposed by Sackmann and Träuble.[7] As per this model, the spin label, when incorporated in liposomes, creates large pockets of free volume around its site of incorporation, particularly below the phase transition temperature (T_m) of the lipid. Even above T_m these pockets of free volume continue to exist. This allows the steroid nucleus to have a high degree of motional freedom whereby the nitroxide undergoes fast tumbling. Owing to these two factors, the EPR spectrum of the spin label SPN in liposomes (Figure 1b) exhibits an intermediate situation of the two extreme cases viz. freely tumbling spin labels in organic solvents and restricted anisotropic motion.

Mode of localization in model membranes

The mode of localization of SPN in lipid bilayers has been determined with the help of NMR experiments. For this purpose, ^1H NMR spectra of liposomes with and without the spin label have been recorded. The assignments of the signals arising from lipid molecules have been made using the data reported in the literature.[14] Since the concentration of the spin label used is low (1 mM), the resonances arising from protons of these molecules are not directly observable. However, it is observed that resonances from different regions of the lipid molecule exhibit line broadening to different extents (Table 1). This indicates that some of the lipid protons are in close proximity (≤ 10 Å) to the paramagnetic nitroxide moiety and therefore experience line broadening on account of dipole–dipole interactions. The results of NMR studies (Table 1) indicate that resonances of terminal methyl and $^+NMe_3$ corresponding to liposomes incorporated with SPN are broader than those of pure liposomes. This clearly shows the definite incorporation of SPN in the interior of the bilayer. The increase in peak width observed for the $^+NMe_3$ resonance could possibly be attributed to the interaction between the hydroxy group of SPN and the polar head

group of the lipid. The increase in the ^{31}P resonance line width (Table 1) also indicates a similar possibility. It is pertinent to note that the hydrogen bonding interaction between the hydroxy group of sterols[15] and an oxygen atom either in the phosphate group[16] or in the ester carbonyl group[17] has been documented in literature. With the hydroxy group of SPN possibly oriented towards the polar head group region of the lipid, the proxyl nitroxide moiety remains in close proximity to the terminal methyl group. This explains the observed broadening of the ^1H NMR signal of the terminal methyl group.

Table 1. Effect of incorporation of spin label SPN on the ^1H NMR and ^{31}P NMR spectra of EPC model membranes (spin label: lecithin, 1:100)

Full width at half maximum ($\Delta \nu_{1/2}$) in Hz			
	^1H NMR		^{31}P
	$^+NMe_3$	Terminal methyl	
Pure EPC ($\nu_{1/2}$)	13.1Ø	26.3Ø	75Ø
EPC+Spin label ($\nu_{1/2}$)$_{LS}$	96.5Ø	122.8Ø	1ØØØ
$\frac{(\Delta\nu_{1/2})_{LS}}{(\Delta\nu_{1/2})_{L}}$	7.3Ø	4.7Ø	1.33

In order to obtain further insight into the mode of localization of SPN within the lipid matrix, ^{13}C spin-lattice relaxation time (T_1) measurements have been carried out. ^{13}C spin-lattice relaxation times are governed by ^1H–^{13}C dipole interaction of the directly bonded C–H vectors in the molecule.[18] This has been utilized to get information regarding regions of immobilization of the molecules incorporated in lipid bilayers.[19] The ^{13}C relaxation times of either the lipids or the externally added molecule can be measured for this purpose. Restricted motion leads to lower T_1 due to shorter spin-lattice relaxation time. In the present case, the effect of the nitroxide moiety in SPN, incorporated into the bilayer, on ^{13}C relaxation times of the lipid molecules are interpreted in terms of the localization of the nitroxide group within the bilayer structure. The effect of the nitroxide group in producing differential changes in T_1 for the carbon nuclei in EPC suggests that such changes are useful in estimating the proximity of the nitroxide moiety to the different carbon atoms of the lipid. We have measured the relaxation times of the carbon atoms of the lipid molecule (Table 2). The assignments of the ^{13}C resonances of EPC have been made using the data reported in the literature.[20]

It may be noted that the observed T_1 values for the chain carbons are the average values for the two chains. The results indicate that the T_1 values of the resonances are generally reduced in the presence of the nitroxide SPN (Table 2). Differential changes in T_1 values for different carbon atoms of the lipid in the presence of SPN, as compared to the corresponding values in pure EPC liposomes, are also observed. The T_1 value of the carbonyl carbon of lipid molecule in the presence of SPN could not be measured, probably because of a very large decrease. Similarly, the T_1 value of the -CH_2OP–choline carbon though measurable, is also reduced to a large extent. This indicates that the hydroxy group of SPN is possibly involved in a hydrogen bonding interaction with the oxygen atom of either the ester carbonyl group or the phosphate moiety. This leads to immobilization and a consequent decrease in T_1 value. As mentioned earlier, such a type of hydrogen bonding interaction has been reported in the literature.[15–17] Within the hydrocarbon chain, the decrease in T_1 is largest for the terminal methyl group. The effect of the nitroxide moiety on the carbon atoms constituting the methylene envelope is complex owing to the differing contributions to the relaxation time from the component resonances and the observed T_1 value is an average one. The decrease in T_1 value observed for the $^+NMe_3$ carbons, though relatively less, may be attributed to the fact that the proxyl nitroxide moiety in SPN is in the flexible side chain which enables it to spend a certain fraction of time near the polar head group of the lipid as well.

Table 2. Effect of SPN on ^{13}C T_1 values of sonicated EPC in D_2O at 30 °C (spin label:lecithin, 1:100)

Carbon	EPC Control T_1(s)	EPC + SPN T_1(s)	% decrease in T_1
Terminal methyl	2.85	1.95	31.6
Methylene envelope	Ø.59	Ø.53	1Ø.2
Olefinic carbon*	Ø.58	Ø.41	29.3
-CH_2OP-choline	2.24	1.ØØ	55.4
Carbonyl carbon	1.ØØ	not observed	-
$^+NMe_3$	Ø.47	Ø.39	17.Ø

*The two resonances corresponding to the olefinic carbons are not sufficiently resolved to determine their relaxation times separately.

Thus, the ^{13}C T_1 results qualitatively map out the localization of SPN in the lipid matrix.

Phase transition studies

The phase transition behaviour of dipalmitoyl phosphatidyl choline (DPPC) dispersions has been studied using the spin label SPN as an EPR sensitive probe. The alterations in the membrane characteristics induced by the drugs mepivacaine, xylocaine and azelaic acid have also been investigated. It is customary to use[21] the empirical parameter h_{+1}/h_0 (the ratio of the heights of the low-field line to the central line in the EPR spectrum of the nitroxide) to follow phase transition characteristics. This parameter (h_{+1}/h_0) has been plotted as a function of temperature (Figure 2). One observes that h_{+1}/h_0 shows an initial gradual increase which is followed by an abrupt, large increase at a particular temperature which corresponds to the transition of lipid hydrocarbon chains from gel to

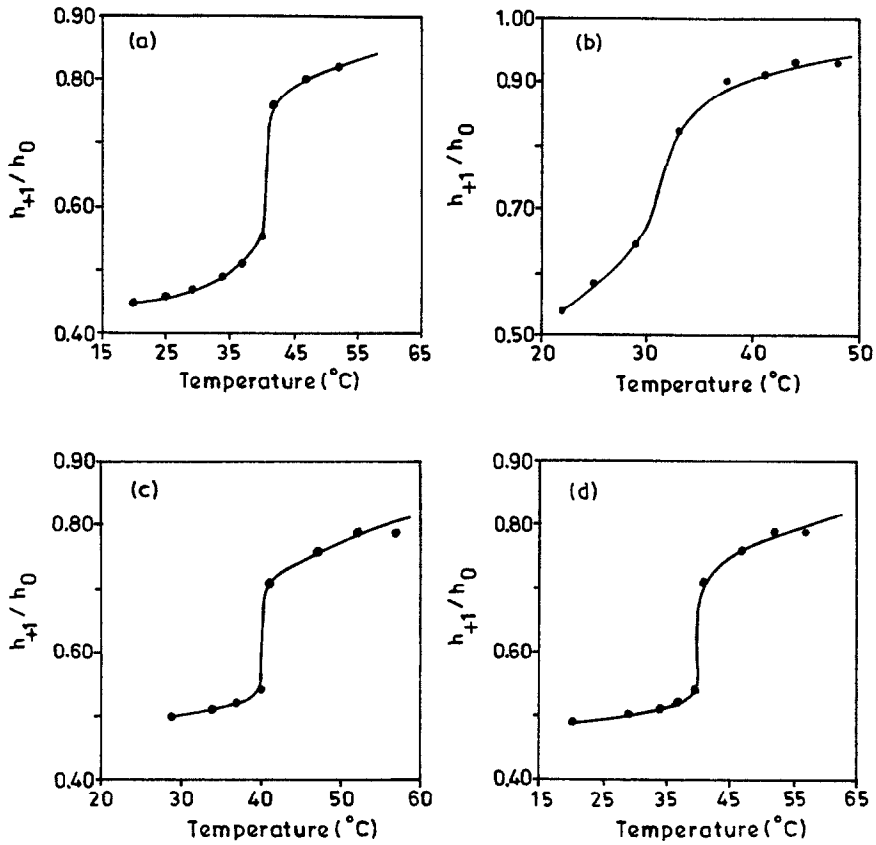


Figure 2. Spectral parameter h_{+1}/h_0, as a function of temperature, (a) pure DPPC (100 mM), (b) DPPC (100 mM) + mepivacaine (40 mM), (c) DPPC (100 mM) + xylocaine (40 mM), and (d) DPPC (100 mM) + azelaic acid (40 mM), using SPN (1 mM) as the spin label

liquid crystalline state. The pre-transition and the main transition temperatures of pure DPPC dispersions thus obtained are 35.5 °C and 41 °C respectively (Table 3). These results are in agreement with the values reported earlier by using other methods[13] as well as by using other spin labels.[15] This conclusively proves that the newly synthesized proxyl nitroxide SPN can be conveniently used for studying phase transition characteristics of liposomes.

Table 3. Phase transition temperatures of DPPC dispersions in the presence of and in the absence of drugs using SPN as the spin label (spin label:DPPC:drug, 1:100:40)

Type of vesicles	Pre-transition temperature(°C)	Main transition temperature(°C)
Pure DPPC	35.5	41.0
DPPC+mepivacaine	not observable	31.0
DPPC+xylocaine	32.0	39.0
DPPC+azelaic acid	32.0	39.5

The curves depicted in Figure 2 exhibit sigmoidal nature of phase transition curves in the presence of as well as in the absence of the drugs. This, in turn, indicates that the presence of these drugs does not alter the cooperativity of phase transition. However, it alters the temperature at which the phase transition occurs. For instance, mepivacaine causes significant lowering of the main phase transition (Table 3). Xylocaine, on the other hand, causes marginal change of the main phase transition temperature. These observations are in agreement with the results of Hubbell *et al.*[22] and Rosenberg *et al.*[23] The incorporation of azelaic acid induces a lowering of the temperature of pre-transition by 3.5 °C and nearly no alteration in the main phase transition temperature. This is in agreement with the results obtained by Bossi *et al.*[13] Thus we conclude that SPN has the ability to report phase transition characteristics of pure lipids as well as of mixed (liposomes incorporated with drugs) systems.

Permeability studies

The permeability of EPC bilayers to ascorbate ions added externally to the aqueous phase has been studied by monitoring the reduction of the nitroxide SPN incorporated in the lipid matrix. The vesicle size homogeneity and the unilamellar nature of the EPC dispersions obtained in the present experiment have been determined. The reduction of the bilayer-fixed spin label has been monitored by observing the changes in the low-field line in the EPR spectrum of SPN as a function of time, as ascorbate ions diffuse through the bilayer. The plots of EPR signal heights $S(t)$ with time (t) using SPN as the spin label have been depicted in Figure 3. The points shown are experimental points through which theoretical curves have been drawn after fitting the data to Eq. 1:

$$S(t) = S_o(0)e^{-k_o t} + S_i(0)e^{-k_i t} \quad (1)$$

where $S(t)$ is the signal height due to total spin label present at time t, $S_o(0)$ and $S_i(0)$ are signal heights due to initial concentration of spin labels present in the outer and the inner monolayer respectively and k_o and k_i are the rate constants for the reduction of the spin labels present in the outer and the inner monolayer respectively. The values of k_o and k_i have been obtained by using Eqs 2 and 3 where r_o and r_i are the radii of the outer and the inner monolayer respectively.

$$S(0) = S_o(0) + S_i(0) \quad (2)$$
$$S(0) / S(t) = r_o^2 / r_i^2 \quad (3)$$

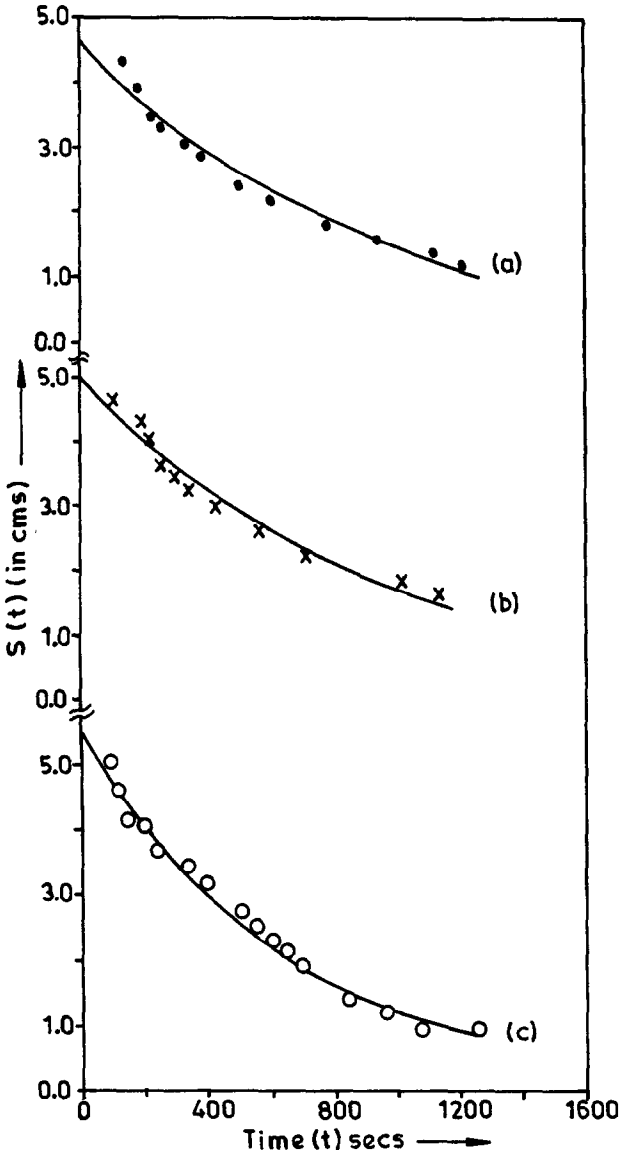


Figure 3. Signal height $S(t)$ of the EPR spectral line of spin label SPN (1.0 mM) vs time. The points shown are experimental points through which theoretical curves have been drawn after fitting the data to $S(t) = S_o(0)e^{-k_o t} + S_i(0)e^{-k_i t}$, where $S_o(0)$ and $S_i(0)$ are respective initial signal heights due to the spin label in the outer and inner monolayers of sonicated vesicles. (a) (●), pure EPC (100 mM); (b) (X) EPC (100 mM) + mepivacaine (40 mM), and (c) (○) EPC (100 mM) + xylocaine (40 mM)

It has been reported[24] that the outer radius of the sonicated vesicles is around 250 Å and the thickness of the bilayer is around 50 Å. Using the methodology described earlier,[15] the values of k_o and k_i are obtained by least square fitting of the data utilizing Eq. 1. The half-life times of reduction of spin labels residing in the outer and the inner monolayers of EPC bilayers have also been determined (Table 4).

Table 4. Half-life times (min) for the reduction of spin label SPN incorporated in EPC bilayers in the presence of different drugs at 30 °C (permeating agent used was sodium ascorbate)

	Pure EPC	EPC + mepivacaine	EPC + xylocaine
Outer monolayer	0.08	0.07	6.01
Inner monolayer	10.30	10.07	8.50

It is pertinent to note that though Kornberg and McConnell[25] observed that ascorbate ions do not penetrate the EPC bilayer at 0 °C, the present permeation experiments have been carried out at 30 °C. This temperature is conducive to the permeation of ascorbate ions through the bilayer. This is well documented in literature.[26] Moreover, it is worthwhile to mention that the time-scales of our permeation experiments are much shorter than that of the flip–flop experiments of Kornberg and McConnell.[25] Since these authors have shown that the asymmetry in the distribution of spin labels between the bilayers decays with a half-life of about 6.5 h at 30 °C, the flip–flop motion from the inner monolayer to the outer monolayer is clearly a very slow process. Hence the flip–flop motion is highly unlikely in the present case and has not been considered in the interpretation of our results.

Analysis of the results of permeability studies reveal that for pure EPC the half-life times for reduction of spin labels present in the outer and inner monolayers are significantly different. This observation could be attributed to the fact that the ascorbate ions when introduced on the outer side of the EPC vesicles, diffuse through the bilayer and reduce the spin labels. The spin labels which reside in the outer monolayer are readily accessible and therefore undergo reduction at a faster rate in comparison to the spin labels in the inner monolayer. Thus, the half-life times for reduction of spin labels in the inner monolayer are longer than those in the outer monolayer. One notices that these values remain unaltered in the presence of mepivacaine. This indicates that the permeability profile of lipid bilayers remains unaltered by incorporation of mepivacaine. The half-life time for reduction of spin labels residing in the outer monolayer in the presence of xylocaine is observed to be higher (6.01 min) in comparison to that for pure EPC vesicles. This could be due possibly to the binding of

xylocaine to the head group of the lipid which, in turn, prevents fast permeation of ascorbate ions. However, a decrease in the half-life time for the inner monolayer (8.57 min) signifies enhanced accessibility of the interior of the lipid matrix to ascorbate ions.

We therefore conclude that the newly synthesized proxyl nitroxide SPN is easily incorporated in the membranes and can be conveniently used as a potential spin probe for studies such as phase transition, permeability, etc. of membranes.

Experimental Section

Materials

L-α-Dipalmitoylphosphatidyl choline (DPPC) was obtained from Sigma Chemical Company, U.S.A. Egg phosphatidyl choline (EPC) was isolated and purified by the method of Keough.[27] Spin label SPN was synthesized in the laboratory (Scheme I). Azelaic acid was obtained from Serva Fienobiochimica, Heidelberg, Germany. Mepivacaine and xylocaine were used as their hydrochloride salts and were purchased from Astra Pharmaceutical Products, Massachusetts, U.S.A. Sodium ascorbate was obtained from Sisco Research Laboratories, India and other reagents used were of analytical grade.

Multilamellar dispersions of lipid used for the phase transition experiments were prepared following Hill's method.[28] Chloroform solutions of appropriate quantities of lipid (100 mM) and spin label (1 mM) were evaporated to dryness under a stream of nitrogen gas. The film was dried under vacuum for 3–4 h and then hydrated with appropriate amount of 10 mM phosphate buffer (pH 7.5) containing the desired amount of drug. The system was allowed to equilibrate for 30 min before vortexing.

For permeation experiments unilameller vesicles were used. Unilamellar vesicles were prepared by sonicating multilamellar dispersions immersed in an ice bath using B-30 sonifier fitted with microtip (Branson Sonic Power Co.) at a duty cycle of 50%. Sonication was carried out until optical clarity was achieved. The preparations were centrifuged for 10 min at 20,000 x *g* (16,000 rpm) using Sorvall RC 5B centrifuge to separate out the titanium particles. Homogeneity of vesicle size of the phospholipid dispersions was analyzed by Sepharose 4B chromatography. Uniformity of vesicle size was checked on electron microscope. Vesicles of radii around 250 Å are formed under such conditions.[24] Electron microscopic studies were conducted using Jeol JEM 100 S electron microscope at a high voltage of 60 kV. The samples were prepared by placing a drop of suspension on a thin film of formvar coated copper slot grid which was allowed to dry before placing under the microscope. Sodium ascorbate (5 mM) was added to the samples just before recording the EPR spectrum ($t = 0$).

Methods

EPR experiments were carried out on an X-band E-112 Varian Spectrometer with a 100 kHz field modulation and detection unit. Samples were taken in 50 μL glass capillaries sealed at one end and mounted in the variable temperature accessory of the Varian E-112 Spectrometer. Temperature could be controlled to an accuracy of ±1 °C using a Varian V-4540 unit. The sample temperature was determined using a copper–constantan thermocouple kept in contact with the sample capillary.

^1H, ^{31}P and ^{13}C NMR spectra of sonicated liposomes in D_2O were recorded using Bruker AM-500 FT-NMR Spectrometer interfaced with an Aspect 3000 computer. The ^{31}P NMR spectra were obtained under proton decoupling and a line broadening of 20 Hz has been employed. In the ^{13}C experiments, a repetition time of 16 s was used. Protons were decoupled using broad band noise decoupling. Spin-lattice relaxation times were measured employing ($180° < \tau < 90°$) pulse sequence at a temperature of 30 °C.

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