Determining the Location of Hydrophobic Spin Traps within Liposomes

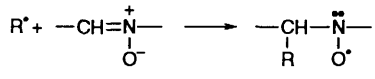
Gila Strul,[a] Hugo E. Gottlieb,[a] Aryeh A. Frimer*,[a] and Lev Weiner[b]
[a] *Ethel and David Resnick Chair in Active Oxygen Chemistry, Department of Chemistry, Bar-Ilan University, Ramat Gan 52900, Israel*
[b] *Department of Organic Chemistry, Weizmann Institute of Science, Rehovot, Israel*

We have recently reported on an initial study of free radical penetration into liposomal membrane using two new hydrophobic spin traps, namely 2,2-dimethyl-4-phenyl-2*H*-imidazole 1-oxide (DMPIO) and its 2-methyl-2-nonyl analogue (MNPIO). The radical trapping data was most readily resolved by positing that only MNPIO is lodged completely within the lipid bilayer; the *N*-oxide group of DMPIO, on the other hand, is actually located at or near the lipid/water interface, available for interaction with aqueous phase radicals. We have been able to confirm these results by using the ^{13}C chemical shifts of these spin traps as a gauge with which to measure the polarity of the microenvironment experienced by the carbons. As expected, the spin traps experience a gradient of solvent polarities, increasing as one goes from deep within the lipid bilayer out toward the aqueous phase.

Oxygen radicals lie at the heart of a life–death nexus. They are formed naturally during 'the breath of life', yet present a constant serious threat to living organisms. As Angier[1] has so eloquently put it: 'Free radicals are the price we pay for breathing, the inescapable byproducts of living in a world full of oxygen . . . We need free radicals to live, but they are also the bane of our existence.' Extensive research over the past two decades has shown that oxygen radicals are important causative agents of aging and a plethora of human diseases.[2,3]

Interestingly, the focus of oxygen radical research has not been limited to aqueous (polar/protic) media. In order to gain insight into the various possible modes of action available to these active oxygen species, the organic chemistry of oxy-radicals in general and superoxide anions in particular has been explored thoroughly even in non-polar aprotic media.[4] Indeed, it has been argued that aprotic media might well mimic the hydrophobic conditions in cell membranes and the active sites of various enzymes.[5] Nevertheless, the validity of these latter assertions has yet to be established. To this end, we recently embarked on a study of the chemical reactions of oxy-radicals with indicative substrates intercalated between the hydrophobic phospholipid bilayers of model biological membranes (liposomes, micelles *etc.*) dispersed in aqueous media.

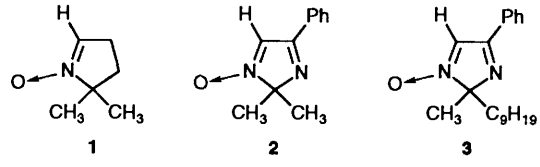
EPR spin trapping techniques have been particularly useful in studying radical processes in biological and biomimetic systems.[6–8] In this approach, nitrone spin traps intercept transient radicals producing, in turn, persistent aminoxyl spin adducts [eqn. (1)]. The EPR parameters of the latter can be



(1)

used to identify the trapped radical. Our goal, then, was to intercalate suitable spin trapping nitrones into the lipid bilayer of liposomes and study the radical activity therein. Considering, however, the polarity and hydrophilicity of nitrones, it is by no means a trivial matter to keep the spin traps within the lipid bilayer.

Indeed, we have recently reported[9] on an initial study of free radical penetration into liposomal membrane using two new hydrophobic analogues of the well-studied 5,5-dimethyl-1-pyrroline 1-oxide (DMPO, **1**), namely 2,2-dimethyl-4-phenyl-2*H*-imidazole 1-oxide (DMPIO, **2**) and its 2-methyl-2-nonyl analogue (MNPIO, **3**).[10] These studies confirm that DMPIO



and MNPIO are both lypophilic: their respective distribution coefficients in an equivolume mixture of octanol and water are 8.5 and $> 10^3$. Nevertheless, the radical trapping data was most readily resolved by positing that only MNPIO is lodged completely within the lipid bilayer; the *N*-oxide group of DMPIO, on the other hand, is actually located at or near the lipid/water interface, available for interaction with aqueous phase radicals. However, because these results were only suggestive as to the specific location of these spin traps—and in particular their reactive nitrone carbon—within the liposome bilayer, we wished to obtain more conclusive evidence.

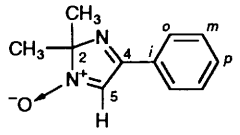
In this regard, we note that several groups have reported a general correlation between the NMR chemical shifts of the various hydrogens and carbons of a given compound and the polarity of the solvent in which the spectrum is measured.[11] From these correlation results, it should be possible to use the ^1H and ^{13}C chemical shifts as a gauge with which to measure the polarity of the microenvironment experienced by the molecule under study in heterogeneous systems, such as micelles or vesicles. We report below on our application of this technique to determine the location of DMPIO and MNPIO within the lipid bilayer of dimyristoyl phosphatidylcholine (DMPC) liposomes.

Results and Discussion

The NMR spectra of DMPIO and MNPIO were measured in four solvents: CCl_4, $CDCl_3$, CD_3CN and CD_3OD (Tables 1 and 2). Because of the hydrophobicity of DMPIO and MNPIO (octanol/water partition coefficient is 8.5 and $> 10^3$, respectively),[9] it was impossible to obtain NMR spectra of either spin trap in a purely aqueous system.

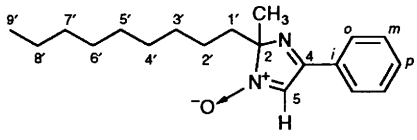
We, indeed, observed that the chemical shifts of the various hydrogens and carbons are correlated with solvent polarity (as measured by $E_{T(30)}$),[12] increasing (*i.e.*, showing a downfield shift) with increasing solvent polarity, but the changes are generally quite small (0–1 ppm for ^1H and 0–2 ppm for ^{13}C).

Table 1 ^1H and ^{13}C NMR assignment for DMPIO



Position	^1H NMR δ		^{13}C NMR δ	
	$CDCl_3$	CD_3OD	$CDCl_3$	CD_3OD
2			102.29	103.03
4			164.73	166.73
5	7.73	8.19	125.26	128.43
i			131.18	132.17
o	7.87	7.98	127.22	128.56
m	7.52	7.56	129.10	130.14
p	7.52	7.53	131.91	133.22
CH_3	1.64	1.60	24.50	24.70

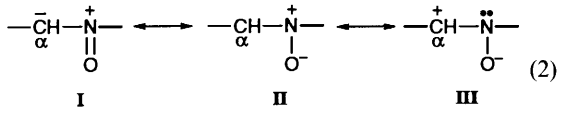
Table 2 ^1H and ^{13}C NMR assignment for MNPIO



Position	^1H NMR δ		^{13}C NMR δ	
	$CDCl_3$	CD_3OD	$CDCl_3$	CD_3OD
2			104.65	105.43
4			165.13	167.30
5	7.74	8.22	126.06	129.39
i			131.10	132.02
o	7.88	7.99	126.06	128.52
m	7.53	7.53	129.03	130.17
p	7.53	7.57	131.79	133.24
CH_3	1.62	1.60	24.18	24.49
1′	1.97, 2.13	1.97, 2.11	37.42	38.12
2′	1.04	1.00	22.58	23.49
3′	*a*	*a*	29.45[b]	30.47[b]
4′	*a*	*a*	29.34[b]	30.36[b]
5′	*a*	*a*	29.21[b]	30.30[b]
6′	*a*	*a*	29.21[b]	30.18[b]
7′	*a*	*a*	31.82	32.98
8′	*a*	*a*	22.64	23.66
9′	0.85	0.86	14.07	14.14

[a] These hydrogens appear as a multiplet in the region δ 1.2–1.3. [b] The assignment of these very close peaks is arbitrary.

The C-2, -4 and -5 nitrone ring carbons are the most sensitive, showing solvent effects of *ca.* 1.5, 3.0 and 5.5 ppm, respectively, in going from CCl_4 to methanol. The large effect observed for the methyne group of the nitrone moiety (C-5) has previously been noted by Janzen and coworkers,[11f] and can be readily explained if we recall that, in NMR spectroscopy, as the electron deficiency ('deshielding') on a carbon or hydrogen increases it is shifted downfield.[13] In the case of the nitrone moiety [eqn. (2)] the α-carbon is most electron deficient in



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charge-separated canonical structure III, whose contribution is expected to increase with solvent polarity.

Plots of the ^{13}C chemical shift of C-2, -4 and -5 of either DMPIO or MNPIO *vs.* solvent polarity give straight lines (Figs. 1 and 2) with very good to excellent correlation coefficients (r^2 = 0.93–0.95 for C-2 and 0.98–0.99 for C-4 and -5; see Table 3).† This chemical shift/$E_{T(30)}$ correlation suggests that if we can determine the ^{13}C NMR data for the carbons of DMPIO or MNPIO intercalated in the bilayer of a liposome, we should have a clear indication as to the polarity of the microenvironment—and, hence, the probable location—of these nitrones in the heterogeneous liposomal systems.

The ^{13}C chemical shift of C-2, -4 and -5 within DMPC

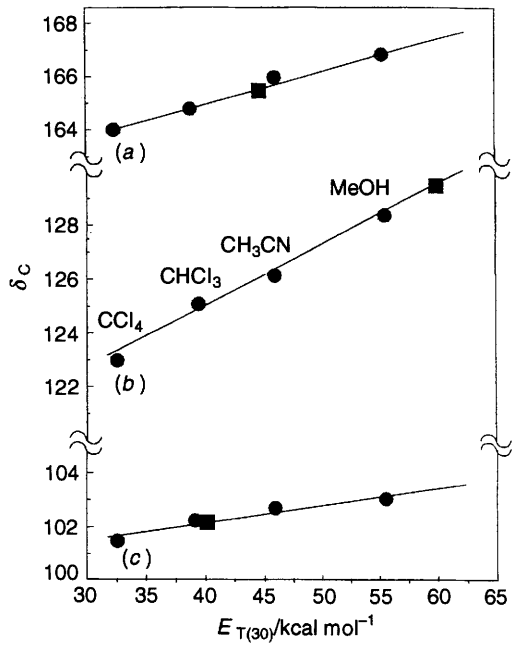

Fig. 1 Plot of ^{13}C NMR chemical shifts of DMPIO *vs.* solvent polarity parameter $E_{T(30)}$[12] for (*a*) C-4, (*b*) C-5 (nitrone) and (*c*) C-2. For the DMPC liposome case (squares), the resulting straight line could be used to determine the microenvironmental polarity of the respective carbons based on the observed ^{13}C NMR chemical shifts.

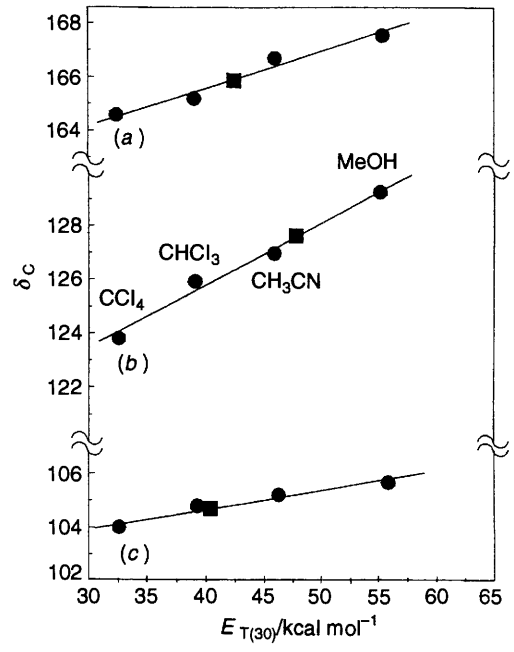

Fig. 2 Plot of ^{13}C NMR chemical shifts of MNPIO *vs.* solvent polarity parameter $E_{T(30)}$[12] for (*a*) C-4, (*b*) C-5 (nitrone) and (*c*) C-2. For the DMPC liposome case (squares), the resulting straight line could be used to determine the microenvironmental polarity of the respective carbons based on the observed ^{13}C NMR chemical shifts.

† Similar plots (not shown) using the nitrone ^1H chemical shifts gave substantially poorer results.

Table 3 ^{13}C NMR chemical shifts (ppm) for DMPIO and MNPIO in pure solvents and dimyristoyl phosphatidylcholine liposomes

Solvent	$E_{T(30)}$ [a]	DMPIO C-2	DMPIO C-4	DMPIO C-5	MNPIO C-2	MNPIO C-4	MNPIO C-5
CCl_4	32.5	101.52	163.98	122.99	103.92	164.53	123.91
$CDCl_3$	39.1	102.29	164.73	125.24	104.65	165.13	126.06
CD_3CN	46.0	102.66	165.97	126.15	105.02	166.47	127.09
CD_3OD	55.5	103.03	166.73	128.43	105.43	167.30	129.39
DMPC Liposomes [b]		102.17	165.42	129.55	104.4	165.6	127.6
(calc. E_T) [c]		(40.0)	(44.3)	(61.4)	(39.5)	(41.0)	(47.5)
Correlation coefficient (r^2) [c]		0.929	0.976	0.978	0.946	0.974	0.985

[a] kcal mol^{-1} At 25 °C; from ref. 12; 1 kcal = 4.184 kJ. The corresponding $E_{T(30)}$ value of H_2O is 63.1. [b] Spectra were normally taken at 25 ± 1 °C. However, since the phase-transition temperature of DMPC liposomes is approximately 22 °C,[15] the spectra of the latter were measured at 31 °C. In the case of MNPIO, the peaks are relatively broad and, hence, the chemical shifts are given to only one decimal place. [c] From Figs. 1 (DMPIO) and 2 (MNPIO).

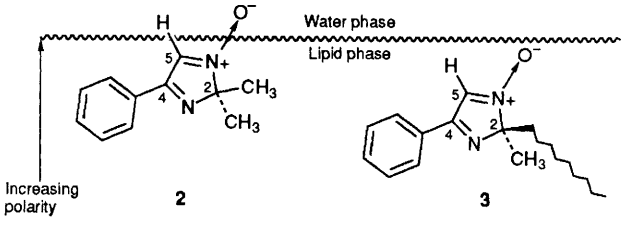

Fig. 3 Pictorial representation of the arrangement of DMPIO and MNPIO within the lipid bilayer

liposomes and the corresponding calculated E_T values are given in Table 3. Looking first at the data for C-5, we see clearly that the nitronyl function of DMPIO has an extrapolated E_T = 61.4 kcal mol^{-1} which lies between methanol (55.5) and water (63.1); hence, the nitrone moiety in this case resides in a highly polar environment of the liposome. The microenvironment of the nitrone function of MNPIO (E_T = 47.5 kcal mol^{-1}), on the other hand, is much less polar—something on the order of acetonitrile (46.0).

Yet, C-5 tells only part of the story. If we turn now to the carbons adjacent to the nitrone moiety (*i.e.* C-2 and -4) of both spin traps, we discover that the calculated E_T for both these carbons place them in environments substantially more lipophilic than that of C-5. More importantly, however, the E_T values of C-2 and -4 are not the same, with the order of increasing solvent polarity felt by the various ring carbons being C-2 < -4 < -5. Thus, the spin traps experience a gradient of solvent polarities. Indeed, a gradient of solvent polarity is expected within the liposome, increasing as one goes from deep within the lipid bilayer out toward the aqueous phase.

The above data suggest the situation represented simplistically by Fig. 3. There is little doubt that the hydrophilic dipolar nitrone group gravitates toward the aqueous layer; indeed, the unsubstituted DMPO is water soluble (octanol/water partition coefficient is 0.9).[9] In the case of DMPIO and MNPIO this hydrophilicity is countered by the hydrophobicity/lipophilicity of the phenyl and/or nonyl substituents (partition coefficient is 8.5 and > 10^3, respectively)[9] which draw the spin traps back toward the centre of the lipid bilayer. The degree of penetration of nitrone group into the water phase depends, then, on the relative strengths of these opposing effects. The E_T data indicate that MNPIO which bears both anchors is held well embedded within the lipid bilayer though its nitrone group reaches out towards more polar environments. This data is also consistent with the relative width of the ^{13}C NMR peaks (about 30 Hz), which suggests that the movement of the spin trap is confined, presumably by the surrounding lipid molecules. In the DMPIO case, the single phenyl anchor is much less effective, and as indicated by the various calculated E_T values, the nitrone group is located at or very near the lipid/water interface. The sharpness of the peaks, essentially equivalent to those observed in pure solvent, suggests free movement. A rapid exchange of DMPIO between polar and non-polar regions of the heterogeneous media may in fact be occurring, as suggested by Janzen;[14] however, prior data[9] seem to rule this out.

In conclusion, then, the results of the present NMR study confirm our previous radical trapping results[9] which also suggested that the reactive site of DMPIO resides at or near the surface of the liposome, enabling it to scavenge assorted radicals (*e.g.* hydroxyl and alkoxyl radicals) generated in the aqueous phase. The contrasting ineffectiveness of MNPIO is consistent with its being located well within the lipid bilayer and hence almost inaccessible to these radicals.

Experimental

Spin traps DMPIO and MNPIO were synthesized at The Institute of Organic Chemistry, Novosibirsk, Russia.[10] Dimyristoyl phosphatidylcholine (DPMC) was obtained from Sigma, while the deuteriated solvents were obtained from Aldrich. Unilamellar vesicles were prepared as previously described.[9] The NMR spectra were recorded on a Bruker AM 300 Fourier transform spectrometer. The DMPC vesicle solutions were studied at 31 °C because the phase-transition temperature for DMPC is close to 22 °C,[15] all other spectra were taken at 25 ± 1 °C. The NMR spectra were generally recorded while locked to the deuterium signals of the respective solvents. The chemical shifts were measured relative to internal tetramethylsilane, except the aqueous vesicle solutions in which a drop of MeOH was added to the sample tube at the end of acquisition. The spectrum was then repeated until the methanol peak at δ 49.5 could be clearly detected. This was used as the reference for the original spectrum. For the spectra in pure solvents, the concentration of the spin traps was 0.1 mol dm^{-3} or less. For the vesicle experiment, the ratio of DMPC to nitrone was 1.8 to 1, with a nitrone concentration of 0.1 mol dm^{-3} in a buffer[9] prepared with 10% D_2O–H_2O.

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