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Active oxygen chemistry within the liposomal bilayer Part IV: Locating 2′,7′-dichlorofluorescein (DCF), 2′,7′-dichlorodihydrofluorescein (DCFH) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) in the lipid bilayer

Michal Afri [a,*], Aryeh A. Frimer [b], Yael Cohen [a]

[a] *The Department of Chemistry, Bar Ilan University, Ramat Gan 52900, Israel*
[b] *The Ethel and David Resnick Chair in Active Oxygen Chemistry, Ramat Gan 52900, Israel*

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Abstract

2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) is commonly used to detect the generation of reactive oxygen intermediates and for assessing the overall oxidative stress in toxicological phenomenon. It has been suggested that DCFH-DA crosses the cell membrane, subsequently undergoing deacetylation by intracellular esterases. The resulting 2′,7′-dichlorodihydrofluorescein (DCFH) is proposed to react with intracellular hydrogen peroxide or other oxidizing ROS to give the fluorescent 2′,7′-dichlorofluorescein (DCF). Using an NMR chemical shift-polarity correlation, we have determined that DCFH-DA and DCFH are located well within the lipid bilayer and certainly not at the interface. These results, therefore, put into serious question the proposed ability of DCFH to come in contact with the aqueous phase and thereby interact with aqueous intracellular ROS and components. However, H_2O_2 and superoxide can cross or at least penetrate the lipid bilayer and react with certain lipophilic substrates. This may well describe the mode of reaction of these and other ROS with DCFH.

Keywords: Liposomes; NMR; Reactive oxygen species; ROS; Lipid bilayer; Membranes; 2′,7′-Dichlorodihydrofluorescein; 2′,7′-Dichlorofluorescin; 2′,7′-Dichlorodihydrofluorescein diacetate; 2′,7′-Dichlorofluorescin diacetate; 2′,7′-Dichlorofluorescein

1. Introduction

2′,7′-Dichlorofluorescein (Scheme 1, **3**, DCF), like other members of the fluorescein family, can be chemically reduced to the corresponding colorless, nonfluorescent leuco dye (Brandt and Keston, 1965; Compton et al., 1988). This "dihydro" derivative, 2′,7′-dichloro*dihydro*fluores*cein* (**2**, DCFH), also known as 2′,7′-dichlorofluores*cin*, is readily oxidized back to the parent dye by various reactive oxygen species (ROS) (LeBel et al., 1992). It can thus serve as a fluorogenic probe for de-

* Corresponding author. Tel.: +972-3-5318323; fax: +972-3-5351250.
E-mail address: afrimi@mail.biu.ac.il (M. Afri).

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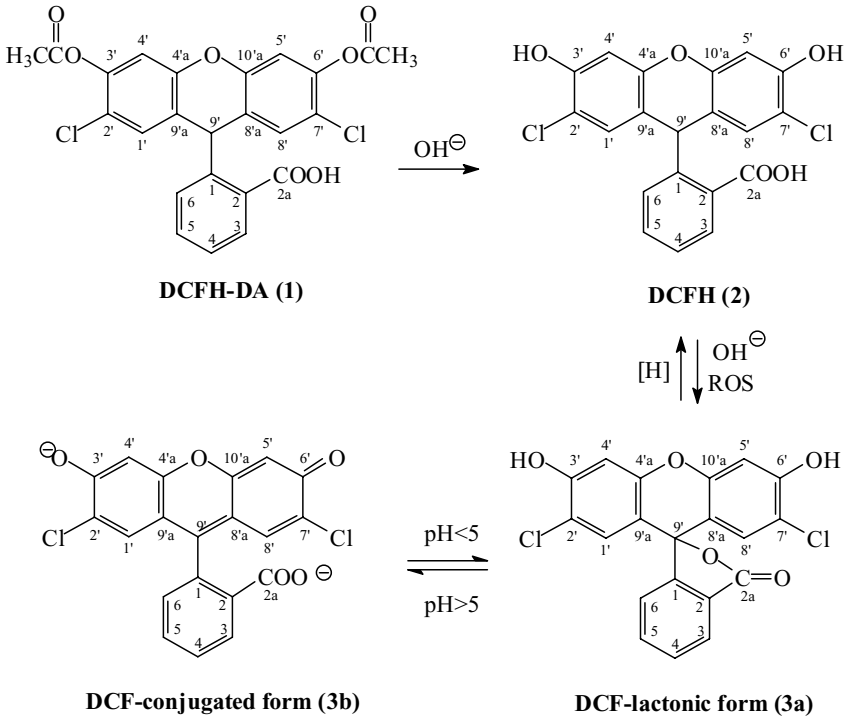


Scheme 1. The interconversion of fluoresceins **1**, **2**, **3a** and **3b**.

tecting oxidative activity in cells and tissues (Bass et al., 1983; Scott et al., 1988)—though, this oxidation may not easily discriminate between the various reactive oxygen species (LeBel et al., 1992; Hempel et al., 1999). A more lipophilic analog of **2**, 2′,7′-dichloro*dihydro*fluores*cein* diacetate (**1**, DCFH-DA), also dubbed 2′,7′-dichlorofluores*cin* diacetate, is commonly used to detect the generation of reactive oxygen intermediates in neutrophils and macrophages (Denizot and Lang, 1986; Vanhee et al., 1993; Miki et al., 1993). Recent investigations suggest that the cell-permeant DCFH-DA may also be extremely useful for assessing the overall oxidative stress in toxicological phenomenon (LeBel et al., 1992; Scott et al., 1988). In the above processes, it has been generally posited that the lipophilic DCFH-DA (**1**) crosses the cell membrane and then undergoes deacetylation by intracellular esterases. The resulting DCFH (**2**) is proposed to react with intracellular hydrogen peroxide or other oxidizing ROS to give the fluorescent compound DCF (**3**) (Rosenkranz et al., 1992; Garn et al., 1994; Manger et al., 1993; Keston and Brandt, 1965; Rota et al., 1999a,b; Wardman et al., 2002).

There is disagreement and confusion, however, regarding the locus of DCFH and DCF action. Most papers (LeBel et al., 1992; Rosenkranz et al., 1992; Marchesi et al., 1999; Oyama et al., 1994) maintain that DCFH, which bears two phenolic groups and a carboxylic acid moiety, is sufficiently polar to enter the aqueous compartment of the cell where it is converted to DCF. By contrast, Royall and Ischiropoulos (1993) have demonstrated that DCFH and DCF are not trapped within endothelial cells, which argues against intracellular processes. Scott et al. (1988) note that they cannot exclude the possibility that the DCFH-DA is deacetylated at or near the cell membrane surface and that the resulting DCFH remains surface-associated during its subsequent oxidation. In this light, we note that DCFH-DA, DCFH and DCF are expected to have very different polarities and lipophilicities, which may well have a strong influence on their average location within the membrane.

In previous works (Strul et al., 1994; Frimer et al., 1996; Weitman et al., 2001; Afri et al., 2002, 2004), we described the application of an NMR technique for determining the location of various molecules within the liposomal bilayer—a simple and convenient model for a cell membrane. This method is based on the good to excellent correlation often observed between ^{13}C NMR chemical shifts (δ) of a polarizable carbon and the polarities of the solvents in which the spectra are measured (Maciel and Ruben, 1963; Ueji and Makamaura, 1976; Menger et al., 1978, 1988; Menger, 1979; Janzen et al., 1989). The solvent polarity is generally expressed in $E_T(30)$ (Reichardt, 1990, 1994) or Δf (Weitman et al., 2001) units. Once a correlation graph for the various carbon nuclei in a substrate is prepared, the substrate can be intercalated within the liposomal phospholipid bilayer and its ^{13}C chemical shift data measured. Using the correlation graph, this liposomal ^{13}C chemical shift data can be correlated with a corresponding polarity value (E_T). However, a gradient of solvent polarity is expected within the liposomal bilayer, increasing as one goes from deep within the lipid bilayer (approaching the polarity of hexane with an $E_T(30)$ of 30.9 kcal/mol) out towards the aqueous phase (approaching the polarity of pure water with an $E_T(30)$ of 63.1 kcal/mol). Thus, the polarity data obtained via the correlation graph gives us a qualitative measure of a substrate's distance from the interface.

In the present study, we decided to implement this NMR technique in the examination of the location within the liposomal bilayer of the three fluorescent fluorescein probes **1**–**3**. Firstly, we wanted to shed light on the above disagreement regarding the locus of DCFH and DCF action. Moreover, because these compounds bear acidic carboxylic and/or phenolic groups, it is quite possible that the active locus of action of these fluorescein probes is pH or solvent polarity dependent—factors which would affect ionization and solvation mechanisms (a similar phenomenon was observed in the case of hypericin; Weitman et al., 2001). Briefly, our results indicate that the lipophilic diester DCFH-DA (**1**) is located in a deep zone of the liposomal bilayer, while DCFH (**2**) lies in a shallower area. DCF (**3**), the most hydrophilic of the three, resides quite close to the interface but still within the lipid bilayer. This data puts into question the proposed ability of DCFH to interact with aqueous intracellular ROS and components, unless the latter (like H_2O_2 and superoxide) penetrate the lipid bilayer.

2. Materials and methods

2.1. General

The NMR spectra were recorded on a Bruker DPX 300 or DMX 600 Fourier transform spectrometer (Bruker, Rheinstetten, Germany). For 1D NMR spectra we used a QNP probe. All 2D experiments (COSY, HMQC, HMBC and NOESY) were run using the programs from the Bruker software library. NMR spectra were generally taken at $25 \pm 1\,^{\circ}C$. The dimyristoylphosphatidylcholine (DMPC) vesicle solutions, however, were run at $45\,^{\circ}C$, above the phase transition temperature (T_C) of DMPC (Zachariasse et al., 1981). Below this temperature, the mobility of the intercalated molecules is low and the resulting NMR absorptions are very broad. Raising the temperature sharpens the peaks but does not seem to affect the chemical shifts. The NMR spectra were generally recorded while locked on the deuterium signals of the respective solvent. The chemical shifts were measured relative to internal tetramethylsilane (TMS), except in the case of the aqueous vesicle solutions in which we calibrated the spectrum according to the trimethylammonium peak at 54.6 ppm.

Deuterated solvents and acetyl-1-^{13}C chloride, were obtained from Aldrich Chemical Company, Milwaukee, WI. Dimyristoylphosphatidylcholine (DMPC), DCF and DCFH-DA were purchased from Sigma Chemical Company, St. Louis, MO. Phosphate buffer (pH 7.0), citric acid buffer (pH 4.0) and zinc were obtained from Merck KgaA, Darmstadt, Germany. Acetic acid was obtained from Frutarom, Haifa, Israel. KH_2PO_4 and KOH were used in the preparation of a 0.1 M phosphate buffer solution (pH 7.8 and containing 10^{-4} M EDTA); the latter was utilized, in turn, to prepare all aqueous solutions, unless otherwise specified. The general procedure for the preparation of DMPC liposomal suspensions for the NMR studies have been previously described (Afri et al., 2004), with the important exception that the ratio of substrate to lipid was 1:10 (9 mol%), and the concentration of substrate in buffer was 0.01 M.

It should be noted that NMR and UV spectral analysis unambiguously verified that the labile DCFH did not undergo autoxidation during the course of the sometimes lengthy NMR measurements.

2.2. Substrates—preparation and spectral data

DCF (**3**) and DCFH-DA (**1**) are known in the literature, but some of their spectral data is lacking; hence, these are included below where appropriate. A DCFH-DA analog enriched with ^{13}C at the ester carbonyls was prepared using acetyl-1-^{13}C chloride, following the general procedure for the esterification as previously described (Wheeler, 1963), and was diluted with the ^{12}C compound in a 1:1 ratio. The latter was incorporated within DMPC liposomes in a substrate to lipid ratio of 1:20 (4.75 mol%) and a substrate concentration of 0.01 M.

2.2.1. 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA, *1*)

^1H NMR (DMSO) δ 7.89 (1H, dd, $J = 8$, 1.5 Hz, H_3), 7.49 (1H, dt, $J = 7.5$, 1 Hz, H_5), 7.38 (1H, dt, $J = 7.5$, 1 Hz, H_4), 7.27 (2H, s, $H_{4'/5'}$), 7.20 (2H, s, $H_{1'/8'}$), 7.07 (1H, d, $J = 7.5$ Hz, H_6), 6.40 (1H, s, H_9), 2.33 (6H, s, Me); ^{13}C NMR (DMSO) δ 168.89 (C_{2a}), 168.02 (C=O), 148.80 ($C_{4'a/10'a}$), 145.83 ($C_{3'}$), 145.26 (C_1), 132.69 (C_5), 131.13 (C_6), 130.07 (C_3), 127.16 (C_4), 129.95 (C_2), 129.76 ($C_{1'}$), 123.09 ($C_{9'a}$), 120.42 ($C_{2'}$), 112.37 ($C_{4'}$), 20.25 (Me).

2.2.2. Synthesis of 2′,7′-dichlorodihydrofluorescein (DCFH, *2*) (Brandt and Keston, 1965)

DCF (**3**; 0.53 g; 1.3 μmol) was dissolved in hot methanol (40 mL), and acetic acid (20 mL) was introduced with continued heating and stirring. Zinc dust (1.5 g; 0.023 mol) was added in three 0.5 g portions with continued heating and stirring over a period of 5 min, during which time the solution turned colorless. The zinc was removed by filtration, and the filtrate was added to 200 mL of water. The resulting white solid was vacuum filtered to give DCFH (0.42 g, 1.04 μmol, 80% yield). The product oxidizes readily to DCF but is relatively stable when stored in the dark and at low temperature (−18 °C).

2.2.3. 2′,7′-Dichlorodihydrofluorescein (DCFH, *2*)

^1H NMR (DMSO) δ 7.78 (1H, dd, $J = 8$, 1.5 Hz, H_3), 7.39 (1H, dt, $J = 8$, 1.5 Hz, H_5), 7.27 (1H, dt, $J = 8$, 1.5 Hz, H_4), 6.93 (1H, s, $H_{1'/8'}$), 6.92 (2H, d, $J = 8$ Hz, H_6), 6.72 (1H, s, $H_{4'/5'}$), 6.10 (1H, s, H_9); ^{13}C NMR (DMSO) δ 162.26 (C_{2a}), 152.47 ($C_{3'/6'}$), 149.07 ($C_{4'a/10'a}$), 146.82 (C_1), 132.15 (C_5), 130.56 (C_6), 129.68 ($C_{1'/8'}$), 129.23 (C_3), 126.39 (C_4), 115.85 ($C_{8'a/9'a}$), 114.61 ($C_{2'/7'}$), 103.74 ($C_{4'/5'}$), 36.35 ($C_{9'}$). HRMS calcd ($C_{20}H_{12}O_5Cl_2$, M^+) 402.0062, obsd 402.0059.

2.2.4. 2′,7′-Dichlorofluorescein (DCF, ***3a***)

^1H NMR (DMSO) δ 11.09 (2H, bs, OH), 8.02 (1H, d, $J = 7.5$ Hz, H_3), 7.82 (1H, dt, $J = 7.5$, 1 Hz, H_5), 7.75 (1H, dt, $J = 7.5$, 1 Hz, H_4), 7.35 (1H, d, $J = 7.5$ Hz, H_6), 6.93 (2H, s, $H_{4'/5'}$), 6.67 (2H, s, $H_{1'/8'}$); ^{13}C NMR (DMSO) δ 168.17 (C_{2a}), 155.06 ($C_{4'a/10'a}$), 151.41 (C_1), 150.02 ($C_{3'/6'}$), 135.80 (C_5), 130.42 (C_4), 128.08 ($C_{1'/8'}$), 125.82 (C_2), 124.99 (C_3), 123.86 (C_6), 116.17 ($C_{8'a/9'a}$), 110.38 ($C_{3'/7'}$), 103.64 ($C_{4'/5'}$), 81.45 ($C_{9'}$). ^1H NMR (MeOH) δ 8.05 (1H, d, $J = 7.5$ Hz, H_3), 7.82 (1H, dt, $J = 7.5$, 1 Hz, H_5), 7.75 (1H, dt, $J = 7.5$, 1 Hz, H_4), 7.25 (1H, d, $J = 7.5$ Hz, H_6), 6.84 (2H, s, $H_{4'/5'}$), 6.62 (2H, s, $H_{1'/8'}$); ^{13}C NMR (MeOH) δ 170.82 (C_{2a}), 156.76 ($C_{4'a/10'a}$), 153.48 (C_1), 152.33 ($C_{3'/6'}$), 136.94 (C_5), 131.65 (C_4), 129.55 ($C_{1'/8'}$), 127.90 (C_2), 126.27 (C_3), 125.28 (C_6), 118.37 ($C_{8'a/9'a}$), 112.32 ($C_{2'/7'}$), 104.89 ($C_{4'/5'}$), 84.10 ($C_{9'}$).

2.2.5. DCF anion (DCF + KOH, ***3b***)

^1H NMR (0.5% KOH in MeOH) δ 8.11 (1H, dd, $J = 7$, 1.5 Hz, H_3), 7.65 (1H, dt, $J = 7$, 1 Hz, H_4), 7.62 (1H, dt, $J = 7$, 1.5 Hz, H_5), 7.21 (1H, dd, $J = 7$, 1 Hz, H_6), 7.12 (2H, s, $H_{1'/8'}$), 6.58 (2H, s, $H_{4'/5'}$); ^{13}C NMR (0.5% KOH in MeOH) δ 176.10 ($C_{9'}$), 173.71 (C_{2a}), 160.10 ($C_{4'a/10'a}$), 158.79 ($C_{3'/6'}$), 140.99 (C_2), 134.13 (C_1), 131.05 (C_3), 130.61 (C_6), 130.51 (C_4), 130.43 (C_5), 129.77 ($C_{1'/8'}$), 129.01 ($C_{8'a/9'a}$), 112.80 ($C_{2'/7'}$), 104.74 ($C_{4'/5'}$).

2.3. Substrates—partition coefficients

Following literature procedures (Pooler and Valenzeno, 1979; Leo et al., 1971), we carried out partition coefficients studies on DCFH and DCF **3b** using equal volumes of octanol and deionized water (pH 6). The results indicated that DCFH was totally partitioned into the octanol, while DCF **3b** was partitioned in an octanol:water ratio of 2:1.

3. Results and discussion

As noted in Section 1, we decided to examine the location of three fluorescent probes within the liposomal bilayer: DCFH-DA (**1**), DCFH (**2**) and DCF (**3**). Our discussion will be divided according to each of these molecules.

*3.1. 2′,7′-Dichlorodihydrofluorescein diacetate (**1**, DCFH-DA)*

This commercial diester derivative contains two acetoxy groups and a carboxylic acid moiety, all substituted on an aromatic ring. Its UV absorption in methanol is $\lambda_{max} = 256$ nm. Because of the symmetry in the tricyclic ring system, there are six pairs of equivalent carbons in DCFH-DA. We measured the ^{13}C NMR of the diacetate compound in both aprotic (dioxane, acetone and acetonitrile) and protic (methanol, ethanol and *t*-butanol) solvents. The assignment of the various carbons was accomplished using a variety of NMR techniques (including HMBC and HMQC). A plot of the chemical shifts (δ) of the various carbons of DCFH-DA in all six solvents versus solvent polarity [expressed by $E_T(30)$ or by Δf] revealed that there were nine carbons—or to be more precise, the *ipso* carbon C_1, and four pairs of equivalent carbons: the two ester carboxyl carbons, $C_{4'a/10'a}$, $C_{2'/7'}$ and $C_{3'/6'}$—which were particularly and reliably sensitive to solvent polarity changes ($\Delta\delta/\Delta E \geq 0.09$). (This cutoff value is based on empirical results from previous studies; Strul et al., 1994; Frimer et al., 1996; Weitman et al., 2001; Afri et al., 2002, 2004.) Unfortunately, such a chemical shift vs. solvent polarity plot gave bad to poor correlation coefficients (ranging from $r^2 = 0.03$ to 0.72 for $E_T(30)$ and $r^2 = 0.05$ to 0.54 for Δf, data not shown). Fig. 1 (dotted line) shows such a plot for the acetoxy carbonyl. However, the data does readily allow for two different lines: one for the points obtained in alcoholic solvents and the other for those measured in the aprotic solvents (see Fig. 1). This two-line plot suggests that DCFH-DA has two or more mechanisms for attaining solvation (e.g., dipole–dipole interaction, hydrogen bonding, etc.) whose extent and involvement depends on the nature of the solvent (Weitman et al., 2001; Afri et al., 2002). Because both $E_T(30)$ and Δf index correlate excellently with the alcoholic solvent chemical shifts ($r^2 = 0.97$–0.99; see Table 1 and Fig. 2), and furthermore, considering the protic nature of the liposomal

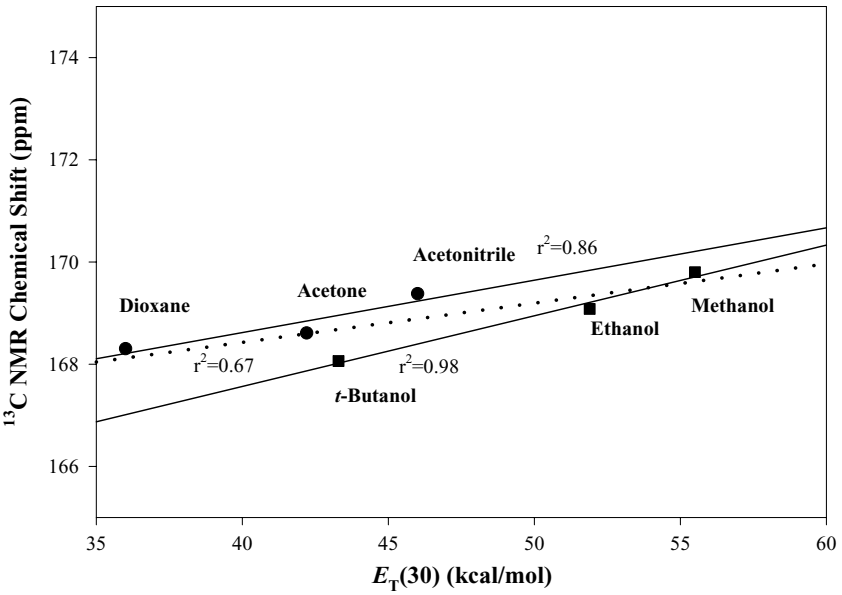


Fig. 1. Plots of the ^{13}C NMR chemical shifts (ppm) for the ester carbonyl of 2′,7′-dichloro*dihydro*fluores*cein* diacetate (DCFH-DA, **1**) vs. solvent polarity [$E_T(30)$]. The filled circles and filled squares represent the aprotic and protic solvent points, respectively. The dotted line is the best fit through all the points. The solid lines represent a two-line fit through the data.

Table 1
^{13}C NMR shifts (ppm) for DCFH-DA (**1**) in pure alcoholic solvents and intercalated within DMPC liposomes

Solvent	$E_T(30)$[a]	Δf	C=O	$C_{4'a/10'a}$	$C_{3'/6'}$	C_1	$C_{2'/7'}$
t-BuOD	43.3	0.2696	168.06	149.60	146.61	146.61	121.77
EtOD	51.9	0.2893	169.08	150.20	147.32	147.13	122.28
MeOD	55.5	0.3095	169.80	150.63	147.72	147.53	122.59
DMPC liposomes[b]			168.37	149.69	146.44	146.44	121.65
Calc. $E_T(30)$, (r^2)[c]			46.0 (0.98)	44.5 (0.98)	41.7 (0.99)	41.0 (0.98)	41.4 (0.99)
Calc. Δf, (r^2)			0.275 (0.99)	0.272 (0.99)	0.261 (0.97)	0.261 (0.99)	0.262 (0.98)

[a] kcal/mol at 25 °C.
[b] Spectra were normally measured at 25 ± 1 °C, except for DMPC liposomes where the spectra were measured at 45 °C.
[c] Error in calculated E_T is ±1 kcal/mol.

environment, we decided to use the protic solvent line equation to locate DCFH-DA within the liposomal bilayer.

The next step was to incorporate the DCFH-DA within the liposome in a substrate to lipid ratio of 1:10 (9 mol%). This ratio was at the limit of NMR detection. We should note in passing that the ^{13}C NMR spectrum of the empty liposome has no absorptions in the region of 70–173 ppm (Frimer et al., 1996). It is in this region that the peaks of interest in DCFH-DA (as well as, DCFH and DCF) fall and are, hence, easily discernable. Unfortunately, the mildly basic conditions (pH 7.8, phosphate buffer), we have normally used in the past, effects the hydrolysis of the ester groups, thereby generating DCFH in situ; the latter is autoxidized in turn to the yellow DCF (UV absorption $\lambda_{max} = 500$ nm). To overcome this problem, we lowered the pH slightly to 7 and obtained clean stable ^{13}C NMR spectra. Table 1 shows the chemical shifts of the nine solvent-sensitive carbons in three alcoholic solvents and in DMPC liposome, using $E_T(30)$ and Δf index. As mentioned above, in both cases, we received straight lines with excellent correlation coefficient of 0.98 (see Fig. 2). Based on this correlation graph we were able to calculate the $E_T(30)$ for polarity of the microenvironment felt by DCFH-DA within the liposomal bilayer. This value appears in Table 1 and summary Table 2. Table 2 also lists

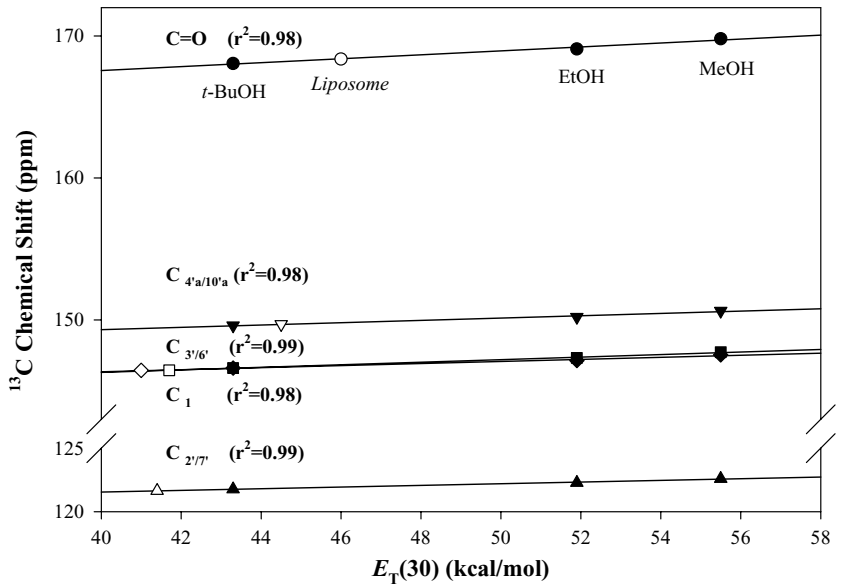


Fig. 2. Plots of the ^{13}C NMR chemical shifts (ppm) for C=O, $C_{4'a/10'a}$, $C_{3'/6'}$, C_1 and $C_{2'/7'}$ of 2′,7′-dichloro*dihydro*fluores*cein* diacetate (DCFH-DA, **1**) vs. solvent polarity [$E_T(30)$].

Table 2
Calculated $E_T(30)$ values for diclorofluorescein derivatives intercalated within the liposomal bilayer

Compound	$C_{2'/7'}$	$C_{3'/6'}$	$C_{4'a/10'a}$	Misc.	$\Delta E_T(30)_{adj}$
DCFH-DA (**1**)	41.4	41.7	44.5	C_1: 41.0; C=O: 46.0	0.3 (2′/7′–3′/6′)
DCFH (**2**)		47.1	45.7		1.4 (3′/6′–4′a/10′a)
DCF-lactone (**3a**)	49.5	49.4			0.1 (2′/7′–3′/6′)
DCF-conjugated (**3b**)	54.7	47.4		$C_{9'}$: 43.7; C_{2a}: 53.0	7.3 (2′/7′–3′/6′)

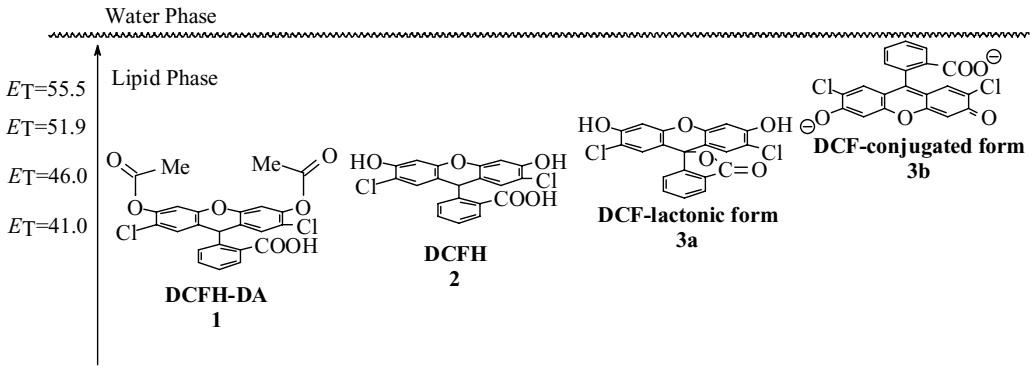
$\Delta E_T(30)_{adj}$—the difference between the $E_T(30)$ of adjacent carbons.

In the case of DCFH-DA, we see that $\Delta E_T(30)_{adj}$ between carbon pairs 2′/7′ and 3′/6′ is only 0.3. Two interpretations of this data are possible. The first is that the DCFH-DA molecule lies in between the lipid chains in a plane essentially parallel to the interface—with all the ring carbons situated in nearly the same polarity region, $E_T(30) \approx 42.5$ kcal/mol. At the time, the ester carbonyls are pulled to a slightly more polar area, $E_T(30) = 46.0$ kcal/mol. This scenario is unlikely, however, since it would require the large DCFH-DA molecule to lie *perpendicular* to the lipid chains, thereby substantially distorting the lipid chain array.

It is more likely, therefore, that the flat DCFH-DA molecule lies in between the lipid chains, in a plane *parallel* to the lipid chains, and that it is in constant rapid rotation in this plane. The timescale of molecular motion is faster than that of the NMR experiment; hence, the values we obtain by the NMR method are actually average locations of substrates within lipid bilayers. Scheme 2 shows the relative average location of DCFH-DA within the liposomal bilayer as compared to DCFH and DCF (vide infra).

Before closing this section, we should note that the common lore in the field of liposome research is that the amount of "intercalant"—substrate incorporated within liposomal bilayers—should not rise above 5 mol%. This is because substantially higher concentrations may affect the integrity of the physical properties of the liposome itself. To verify that the mole percentage has little effect on the location of the substrate within the lipid bilayer (Afri et al., 2004), we prepared DCFH-DA (**1**) which was enriched with ^{13}C in the ester carbonyls, and then incorporated within DMPC liposomes in a substrate to lipid ratio of 1:20 (4.75 mol%) and a substrate concentration of 0.01 M. The calculated $E_T(30)$ values obtained for the labeled carbons were exactly the same! This experiment clearly indicates that higher substrate to lipid ratio of 1:10 has little effect on the location of intercalants within the lipid bilayer.

We should note, however, that even at a concentration of 4.75 mol% phase separation might take place with domains of heterogeneous lipid/DCFH-DA composition resulting (Villalain et al., 1986; Micol et al., 1990). Under these conditions, ^{13}C NMR may reflect average signals from DCFH-DA molecules located at different positions in the membrane. Resolution of this



Scheme 2. Pictorial representation of the average location and orientation of dichlorofluoroscein derivatives within the liposomal bilayer.

possibility is beyond the scope of this paper and is presently under investigation.

3.2. 2′,7′-Dichlorodihydrofluorescein (2, DCFH)

For the purpose of this research, it was necessary to find a good synthesis of DCFH. The conventional method (Cathcart et al., 1983; Tran et al., 2002) involves the saponification of DCFH-DA under basic conditions. The problem with this approach is that under these basic conditions DCFH itself is transformed to DCF via base catalyzed autoxidation (Sosnovsky and Zaret, 1970; Frimer, 1989). This, of course, places data and results obtained using this common saponification method in serious question. We, therefore, turned to a reductive approach in which commercial DCF is reduced with zinc and acetic acid (Brandt and Keston, 1965). The resulting DCFH was sufficiently stable in the dark and low temperature (−18 °C) to permit its spectroscopic (^{1}H NMR, ^{13}C NMR and UV) characterization *for the first time*. The distinctive absorption for DCFH is the H-9′ singlet at 6.21 ppm in the ^{1}H NMR spectra, and the $C_{9'}$ chemical shift at 38.33 ppm in ^{13}C NMR. This colorless compound has a UV absorption at 285 nm, approximately 30 nm higher than the diacetate.

In the next stage we measured the ^{13}C NMR of this molecule in three alcoholic solvents: *i*-propanol, ethanol and methanol. Two pairs of equivalent carbons, $C_{3'/6'}$ and $C_{4'a/10'a}$, proved to be particularly solvent sensitive. There is an excellent correlation ($r^2 = 0.99$) between the chemical shifts and the solvents polarities ($E_T(30)$ and Δf index). As in the case of DCFH-DA, liposomes were prepared using a pH 7 phosphate buffer, since the pH 7.8 alkali buffer induced autoxidation of DCFH to DCF in a matter of minutes. In the liposomal DCFH spectrum we could identify two pairs of equivalents carbons $C_{3'/6'}$ and $C_{4'a/10'a}$, with chemical shifts of 153.04 and 149.94 ppm, respectively. The calculated values of $E_T(30)$ parameters are 47.1 kcal/mol for the former pair and 45.7 kcal/mol for the latter. The $\Delta E_T(30)_{adj}$ for the carbon pairs 3′/6′ and 4′a/10′a is only 1.4. This might suggest that DCFH lies essentially horizontally to the interface. However, as in the case of DCFH-DA, we believe it more likely that the flat DCFH molecule lies in between the lipid chains, in a plane parallel to the lipid chains, and that it is in constant rapid rotation in this plane. In such a dynamic situation, the NMR technique describes the approximate average location of substrates within the lipid bilayer. Scheme 2 depicts the relative average location of DCFH location within the liposomal bilayer; it is somewhat above DCFH-DA, but still far from the interface. This is consistent with our octanol/water partition coefficient studies which indicate that DCFH is totally portioned into the organic phase.

3.3. 2′,7′-Dichlorofluorescein (3, DCF)

Like fluorescein (Klonis et al., 1998; Anthoni et al., 1995), DCF exists in two tautomeric forms depending on pH (see Scheme 1). Under basic conditions, the dominant form is the open form (**3b**), which is fully conjugated across the tricyclic system and possesses a distinctive UV absorption at $\lambda_{max} = 500$ nm and a fluorescence excitation/emission at $\lambda = 395$ nm/450 nm. Under neutral or acidic conditions, one observes ring closure and the formation of the lactonic form (**3a**), which is not fully conjugated and lacks the typical UV absorption and fluorescence. ^{13}C NMR also allows one to distinguish between the two forms based on the chemical shift of $C_{9'}$: in **3b**, $C_{9'}$ is at the end of an extended enone system with a chemical shift at low field ($\delta = 176$ ppm); by contrast, in **3a**, $C_{9'}$ is connected to the lactone oxygen with an upfield resonance ($\delta = 84$ ppm).

We measured the ^{13}C NMR of lactonic DCF (**3a**) in seven organic solvents, four aprotic (acetone, dimetylformamide, dimethylsulfoxide and acetonitrile) and three protic (methanol, ethanol and *t*-butanol) solvents. We found that the chemical shifts of two pairs of equivalent carbons $C_{2'/7'}$, $C_{3'/6'}$, as well as the quaternary carbons $C_{9'}$ and C_{2a} showed substantial sensitivity to solvent polarity ($\Delta\delta/\Delta E \geq 0.09$). In attempting to correlate chemical shifts with solvents polarity, we discovered that here too—as in the case of DCFH-DA and DCFH—two different lines were appropriate for the alcoholic and aprotic organic solvents. The fit in the alcoholic graph was excellent ($r^2 = 0.99$). For the aprotic solvents, however, plots using $E_T(30)$, surprisingly, did not give straight lines at all (data not shown). This leads us to conclusion, that we must use the calibration graphs of the alcoholic solvents, which have hydrogen bonding and the same mechanism of solvation throughout.

It was now time to prepare liposomes intercalated with the lactonic tautomer (**3a**). Initial experiments made it clear that using the usual phosphate buffer (pH 7.8) would yield the conjugated tautomer (**3b**). Hence, we prepared the desired liposomal solution by dispersing DMPC lipid and DCF in citric acid buffer (pH 4). We succeeded in identifying the absorptions corresponding to equivalent carbons $C_{2'/7'}$ and $C_{3'/6'}$ but not those for quaternary carbons $C_{9'}$ and C_{2a}. The calculated values of $E_T(30)$ corresponding to the chemical shifts of $C_{2'/7'}$ and $C_{3'/6'}$ is $E_T(30) \approx$ 49.5 and 49.4 kcal/mol, respectively (see Table 2). The $\Delta E_T(30)_{adj}$ is only 0.1. This again suggests that in DCF **3a**, as in the case of DCFH-DA and DCFH, these carbon pairs experience on average the same polarity. Scheme 2 depicts the location of DCF **3a** within the liposomal bilayer; it is somewhat above DCFH-DA (**1**) and DCFH (**2**), but still well within the liposomal bilayer.

We now turn to the conjugated form of DCF, **3b**. For this purpose, we measured the ^{13}C NMR in alcoholic solvents (*i*-propanol, ethanol and methanol) containing approximately 0.5% KOH. The addition of the base resulted in the instantaneous change in the color of the DCF solution from bright yellow to dark red. This would correspond to deprotonation of one of the phenols of the unconjugated lactone form of DCF (**3a**) and its transformation to the conjugated dienone form (**3b**). The correlation between the chemical shifts and the solvent polarity was outstanding for both solvent polarity parameters.

As mentioned previously, dispersion of DCF and DMPC in basic buffer (pH 7.8) yielded a liposomal solution with intercalated DCF **3b**. In this case, many of the signals are broad reflecting the presence of some dynamic process, presumably a lipid-water exchange (vide infra). Despite the difficulties, we succeeded in distinguishing six carbons (two singles and two pairs): $C_{9'}$, C_{2a}, $C_{3'/6'}$ and $C_{2'/7'}$. The calculated $E_T(30)$, from the line equations, are 43.7, 53.0, 47.4 and 54.7 kcal/mol, respectively. Focusing on the large polarity difference between the adjacent carbons $C_{3'/6'}$ and $C_{2'/7'}$ ($E_T(30) = 47.4$ and 54.7 kcal/mol, respectively), it is clear that this molecule indeed lies parallel to the lipid chains (perpendicular to the interface), with the carboxyl group ($E_T(30) = 53.0$ kcal/mol) anchored not far from the interface.

Since DCF **3a** is located at an $E_T(30) \approx 49.5$ kcal/mol, we might conclude that the dionized form **3b** is certainly more hydrophilic with an E_T value of up to 54.7 kcal/mol. Indeed, London reports (Kachel et al., 1998) that ionized compounds lie closer to the interface than similar non-ionized analogs. We should note, however, that the water/octanol partitioning studies indicate that DCF is partitioned in an octanol:water ratio of 2:1. These results as well as the aforementioned line broadening, strongly suggest that a dynamic water–lipid exchange is occurring and that, hence, the liposomal $E_T(30)$ values obtained for the dionized DCF **3b** is in fact an average over the two environments. It is therefore difficult to be more exact about the location of **3b**.

4. Conclusion

We have noted in Section 1 that a gradient of solvent polarity is expected within the liposomal bilayer, increasing as one goes from deep within the lipid bilayer (approaching the polarity of hexane with an $E_T(30)$ of 30.9 kcal/mol) out towards the aqueous phase (approaching the polarity of pure water with an $E_T(30)$ of 63.1 kcal/mol). Thus, the polarity data obtained via the correlation graph gives us a qualitative measure of a substrate's distance from the interface. Our results indicate that the lipophilic diester DCFH-DA (**1**) is located in a deep zone of the liposomal bilayer [$E_T(30) \approx 42.5$ kcal/mol], while DCFH (**2**) lies in a relatively shallower area [$E_T(30) \approx 46.4$ kcal/mol]. DCF (**3**) is clearly the most hydrophilic of the three, but its location depends on the pH of the medium. Under acidic conditions, DCF exists in the lactone form residing a bit above DCFH [$E_T(30) \approx 49.5$ kcal/mol] but still within the lipid bilayer. Under basic conditions, the ionized form **3b** of DCF resides closer to the interface and probably leaches into the aqueous media.

What is of central importance, however, is that this data locates DCFH well within the lipid bilayer and certainly not adjacent to the interface. We have noted above that much of the literature data on DCF is based on preparations of DCFH in situ via a saponification route. However, under these basic conditions DCFH itself is transformed to DCF via base catalyzed autoxidation This, of course, places data and results obtained using this common saponification method in serious

question. These results also place into doubt the proposed ability of DCFH to come in contact with the aqueous phase and thereby interact with aqueous intracellular ROS and components. We note, however, that our previous work on H_2O_2 (Frimer et al., 1983; Frimer, 1985) and superoxide (Afri et al., 2002) indicates that these two ROS can cross (in the case of H_2O_2) or at least penetrate (in the case of superoxide) the lipid bilayer and react with certain lipophilic substrates. This may well describe the mode of reaction of these and other ROS with DCFH.

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