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Use of merocyanine (MC540) in quantifying lipid domains and packing in phospholipid vesicles and tumor cells

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The fluorescent probe merocyanine (MC540) reports qualitatively on several membrane events. Here we demonstrate that MC540 fluorescence can quantify the degree of coexisting liquid-crystalline and gel states in mixed monotectic phosphatidylcholine (PC) bilayers. The probe exhibits disparate fluorescence wavelength maximas and intensities when incorporated into liquid-crystalline and gel state membranes. The fluorescence measurements parallel partitioning of the EPR spin probe TEMPO between the aqueous environment and the membrane fluid phase. While both techniques can accurately assess the phase transition of synthetic PCs, only MC540 can distinguish between liquid-crystalline phases of different composition. MC540 fluorescence for single-component PC bilayers correlates quantitatively with estimates of the area/molecule determined from surface area/pressure isotherms of lipid monolayers, whereas partitioning of TEMPO fails to assess the relative degree of lipid packing in various fluid state membranes. Additionally, MC540 fluorescence characterizes the interaction of cholesterol with membranes made from condensable (18:0,18:1-PC) and non-condensable (18:0,22:6-PC) lipids. Finally MC540 distinguishes tumor cell membranes differing only in the amount of docosahexaenoic acid (DHA). Thus we conclude that MC540 can be used quantitatively to study phospholipid packing and membrane phases with lipid vesicles and to sense subtle differences in the arrangement of phospholipids in biological membranes.

Introduction

The fluorescent probe merocyanine (MC540) has been used to investigate membrane surface properties of a number of different cells. It was first used to detect action potentials in nerves [1] but was later extended to investigate membrane potentials of mitochondria [2], cardiac cells [3] and chromatophore membranes [4]. More recently its use has expanded to include recognition of 'discrete domains' in the plasma membranes of leukemia cells [5], hematopoietic stem cells [6], immature erythroid cells, macrophages [7] and sperm cells [8]. It has been reported that MC540 can distinguish between what are likely subtle differences in the plasma membranes of very similar cells. For example, leukemic and non-leukemic lymphocytes [5,8] as well as various lines of cytotoxic T lymphocytes and of their sensitive target cells [9] can be distinguished by the cells' interaction with MC540.

Clues as to the mode of action of MC540 have come from investigations with phospholipid vesicles. It has been proposed that in membranes MC540 resides "slightly above the domain of the glycerol backbone of neutral and charged phospholipids" [10] and it is here the dye is very sensitive to structural alterations in the lipids. MC540 binds preferentially to membrane liquid-crystalline phase where its fluorescence intensity is greatly enhanced. Upon the membrane's phase transition to the gel state, MC540 fluorescence decreases substantially. In fact MC540 can be used to detect phase transition temperatures of bilayers composed of

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Abbreviations: BSA, bovine serum albumin; DHA, docosahexaenoic acid; DLPC, dilauroylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; LUV, large unilamellar vesicle; MC540, merocyanine 540; MLV, multilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PBS, phosphate-buffered saline; SUV, small unilamellar vesicle; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

synthetic disaturated phospholipids [11]. Addition of cholesterol to liquid-crystalline state dilauroylphosphatidylcholine bilayers excluded MC540 from the membrane resulting in a substantial decrease in fluorescence. This observation and the fact that MC540 fluorescence is much greater with small unilamellar vesicles containing loosely packed outer leaflet lipids than it is with large unilamellar vesicles with more tightly packed outer leaflet lipids led to the proposal that MC540 can "sense the degree of packing of membrane lipids" [12]. While the interaction of MC540 with membranes is multifaceted, it is now evident that this probe can be used to assess the state of lipids in membranes and perhaps to distinguish subtle differences in membrane structure.

We have been investigating how incorporation of the omega-3 fatty acid docosahexaenoic acid (DHA, $22:6^{\Delta 4,7,10,13,16,19}$) into tumors alters their membrane structure perhaps making the tumors more susceptible to killing by the host's immune system. With six double bonds, DHA is the most unsaturated lipid found in nature. Since the presence of even one double bond greatly effects the structure and function of a fatty acid found in a membrane, it seems reasonable that the large number and location of the fatty acyl unsaturations associated with DHA would impart a unique structure and hence altered function to membranes containing DHA [13]. Our prior experiments have demonstrated that lipid bilayers and tumor cell plasma membranes which had been enriched with DHA are more permeable to several solutes than are non-modified membranes [14]. While DHA-induced permeability is substantial, using fluorescence polarization of DPH and anthroyloxy stearic acids, we were not able to detect significant differences in membrane fluidity caused by DHA compared to that caused by the shorter chain omega-3 fatty acid α-linolenic acid ($18:3^{\Delta 9,12,15}$) [15]. This latter observation may reflect insensitivity of the fluorescent probes, since more recently we have substantiated differences by ^{2}H-NMR in molecular ordering between membranes prepared from more similar phospholipids, the positional isomers [$^2H_{31}$]16:0,α- 18:3-PC and [$^2H_{31}$]16:0,γ-18:3-PC [16]. We have shown that both lipid bilayers and biological membranes enriched in DHA behave differently than those free of DHA and hypothesize the existence of localized DHA-rich domains which may therefore exhibit exceptional 'fluidity'.

In the experiments reported here MC540 is used to quantify coexisting liquid-crystalline and gel states for bilayers containing DHA, to quantitatively measure the degree of lipid packing in these bilayers and to measure the interaction of the membrane acyl chains with cholesterol. The probe is then used to distinguish between T27A tumor cells enriched to different extents with DHA.

Materials and Methods

Materials

Merocyanine 540 was purchased from Sigma, St. Louis, MO. The phospholipids DPPC (16:0,16:0-PC), 18:0,18:1-PC, 18:1,18:1-PC, 18:2,18:2-PC and α-18:3,α-18:3-PC were purchased from Avanti Polar Lipids, Alabaster, AL. All other phospholipids (18:0,18:2-PC, 18:0,α-18:3-PC, 18:0,22:6-PC and 18:0,22:6-PE) were synthesized in our laboratory [15] by the methods of Keough et al. [16] as modified by Comfurius and Zwaal [18]. The EPR probe TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) was purchased from Molecular Probes, Eugene, OR.

Lipid vesicles

Appropriate phospholipids, dissolved in $CHCl_3$, were dried under nitrogen and then overnight in vacuum. Multilamellar vesicles (MLVs) were made by hydrating the lipids above their phase transition temperatures (room temperature for all of the pure unsaturated lipids and 50°C for the DPPC-containing lipids) in a buffer of 0.01 M Tris/0.01 M Na acetate (pH 7.4). Large unilamellar vesicles (LUVs) were made from the MLVs by extrusion 10 times through a 0.1 mm Nucleopore Filter using a temperature controlled Extruder (Lipex, Vancouver, BC, Canada). MLVs were used for the EPR experiments and LUVs for the fluorescence experiments. Small sonicated vesicles (SUVs) were used for the T27A tumor cell fusions. The SUVs were composed of 18:0,22:6-PE/18:0,22:6-PC (1:3) and were prepared by ultrasonic radiation of MLVs on ice at level 5 using a Heat Systems W-380 Cell Disruptor.

Fluorescence

Appropriate quantities of LUVs were mixed with MC540 to give an MC540 concentration of 10 mg/ml and lipid concentrations of 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 mg/ml (for DPPC). Although spectra were obtained for each of the lipid concentrations listed, the MC540/lipid ratio used for the presented data was 0.01. After incubating at the desired temperature for 30 min (30°C for most experiments), fluorescence emission spectra (excitation 540 nm) were obtained on a temperature controlled ($\pm$0.1°C) Perkin-Elmer MPF-66 Fluorescence Spectrophotometer interfaced to a Perkin-Elmer 7700 Professional Computer and run by CLS software. Polarizers were not employed.

Langmuir trough

Pressure/area isotherms were obtained for the phospholipid monolayers at 35°C on a KSV Minitrough using a Wilhelmy Plate. Lipids were added with triple distilled benzene as the carrying solvent (1 mg lipid/ml benzene). The aqueous subphase was 10 mM Na citrate, 100 mM NaCl (pH 5.6). Ultra pure water (de-

ionized, glass distilled and then run through a Milli-Q Plus Water System (Millipore, Bedford, MA)) was used throughout. Compression rates were 1 mN/m per min. Each lipid was run at least three times and the standard error of the mean was less than 2 Å^2.

EPR

The spin probe TEMPO was used to monitor coexisting liquid-crystalline/gel states with monotectic 18:0,22:6-PC/DPPC bilayers. The probe was also used to distinguish between various liquid-crystalline state bilayers composed of symmetric diacyl chain PCs and mixed acyl chain PCs. TEMPO is known to partition preferentially into the membrane liquid-crystalline state [19]. The high-field line resolves into components from TEMPO dissolved in water and membrane, from which the parameter

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

was calculated on the basis of their respective signal heights, P and H. F therefore is directly related to the partition coefficient for TEMPO between the liquid-crystalline phase and water although the exact calculation would require double integration of the first derivative spectra due to the different linewidths of the two components. The spectra were recorded on an IBM/Bruker ER200D x-band EPR spectrometer operating at 9.2 GHz. The spectrometer is interfaced and controlled by a Hewlett Packard 9816 computer system. Spectral parameters were: microwave power 12 mW, sweep width 80 G, sweep time 160 s, modulation amplitude 0.5G, time constant 500 ms, and data set 2000 pts. The experiments were run at room temperature (25 ± 1°C), while the concentration of the samples was 70 mM phospholipid in the presence of 0.1 mM TEMPO.

T27A tumor cells

T27A is a murine non-B, non-T leukemia cell line which was grown in suspension culture in RPMI 1640 medium with antibiotics, 25 mM Hepes, 2 mM glutamine and 20% bovine calf serum (pH 7.2). The cells were cultured at 37°C in a humidified incubator with 7.5% CO_2 and harvested in log phase by centrifugation. Samples containing thirty million cells were then incubated for 1 h at 37°C with various amounts of 18:0,22:6-PE/18:0,22:6-PC (1:3). The lipid modified cells were then washed first with PBS (phosphate buffered saline) + 10% BSA (bovine serum albumin) and then three times with PBS + 10% fetal calf serum to remove the unfused lipid vesicles. The modified cells were then resuspended in PBS + MC540 and their viability determined by Trypan blue exclusion. After incubating with MC540 for 30 min fluorescence emission spectra were recorded.

Gas chromatography

Uptake of DHA into the T27A cells was quantified by gas chromatography. Cell lipids were extracted by $CHCl_3/CH_3OH$ (2:1, v/v) and oxidation prevented by inclusion of 0.2% butylated hydroxytoluene. Saponification was by refluxing for 1 h in 0.5 M NaOH in methanol. Fatty acids were esterified by addition of BF_3-methanol and refluxing again for an additional 30 min. Methyl esters were extracted three times in hexane and the combined extracts reduced to a small volume. The fatty acyl methyl esters were separated on a SRI Gas Chromatograph equipped with a flame ionization detector and interfaced to an IBM 386 Computer. The column was packed with 10% SP-2330 on 100/120 Chromosorb WAW. The temperature ramp was from 190°C to 225°C.

Results

MC540 was used to quantify the extent of co-existing liquid crystalline and gel states in lipid bilayers. Mixed PC large unilamellar vesicles were incubated with MC540 and the fluorescence emission spectra taken. The bilayers were composed of different mixtures of DPPC and either 18:0,18:1-PC or 18:0,22:6-PC. Membrane compositions varied from 100% DPPC/0% mixed chain PC to 100% mixed chain PC/0% DPPC. At the temperature the spectra were taken (30°C) DPPC is in the gel state (T_m = 41.3°C) while the mixed chain PCs are in the liquid-crystalline state (18:0,18:1-PC T_m = 6.3°C [20]; 16:0,22:6-PC T_m = −5°C [21]). For monotectic lipids the extent of liquid-crystalline and gel states would be precisely defined by the lipid molar composition.

Partitioning of the spin probe TEMPO between water and the membrane liquid-crystalline phase confirms that the lipid mixtures used for vesicle formation are indeed monotectic. Fig. 1 demonstrates that a plot of TEMPO partition coefficient (F) vs. the percent liquid-crystal component (18:0,22:6-PC) in the 18:0,22:6-PC/DPPC mixed bilayers is linear (Pearson's correlation, $P < 0.001$).

We next investigated if MC540 could be used to quantitate the extent of co-existing liquid-crystalline and gel states in lipid vesicles composed of the mixed chain PC/DPPC bilayers. In Fig. 2 are representative spectra of MC540 in the absence of phospholipid vesicles (aqueous spectra) and in the presence of phospholipid vesicles composed of 100 mol% DPPC; 10 mol% 18:0,18:1-PC/90 mol% DPPC; 20 mol% 18:0,18:1-PC/80 mol% DPPC; and 30 mol% 18:0,18:1-PC/70 mol% DPPC (Panel 1) and of 100 mol% DPPC; 10 mol% 18:0,22:6-PC/90 mol% DPPC; and 20 mol% 18:0,22:6-PC/80 mol% DPPC (Panel 2). The aqueous spectrum has low fluorescence with a wavelength maximum of about 575 nm. In the presence of 100 mol%

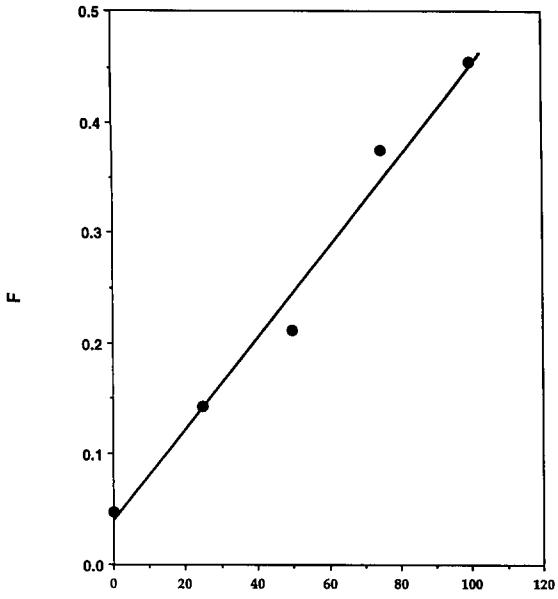

Fig. 1. Partition coefficient F of the spin probe TEMPO as a function of the % of liquid-crystalline state present in 18:0,22:6-PC/DPPC bilayers. At 25°C the percentage of liquid-crystalline state was precisely defined by the mol ratio of the liquid-crystalline (18:0,22:6-PC) to the gel state (DPPC) component.

DPPC bilayers (gel state) the emission wavelength maximum is shifted to about 625 nm. As the percentage of liquid-crystalline component (18:0,18:1-PC or 18:0, 22:6-PC) in the membranes increases, a new fluorescence emission peak emerges with a maximum between 593 and 600 nm. The DHA-containing bilayers exhibit a much greater fluorescence at the same membrane mol% than do the oleic acid-containing bilayers. This probably reflects a difference in lipid packing between the two mixed chain PCs (discussed below).

The ratio of the intensity of the liquid-crystalline peak maximum to that of the gel state maximum can be used as an indicator of the relative amount of liquid-crystalline component in the membranes. Plots of liquid-crystalline fluorescence emission wavelength maximum to gel state fluorescence maximum (lc/gel) vs the percentage of liquid-crystalline state in the bilayers is linear through 50 membrane mol% for 18:0,18:1-PC and about 30 membrane mol% for 18:0,22:6-PC (Fig. 3). The much larger fluorescence emission intensity measured for the DHA-containing bilayers compared to the oleic acid-containing bilayers makes it more difficult to accurately measure the gel state intensity as it is buried under the liquid-crystalline state component.

Another direct observation of liquid-crystalline and gel states would involve measurement of a phase transition in a synthetic, disaturated phospholipid bilayer. We used MC540 to accurately monitor the phase transition of a DPPC membrane. As the membrane melted the fluorescence intensity of the liquid-crystalline component increased dramatically (Fig. 4).

We next used MC540 to quantify the interaction of cholesterol with oleic acid and DHA-containing phospholipids. From prior reports [12,22], we predicted that when cholesterol is added, MC540 would be excluded from bilayers composed of 18:0,18:1-PC (its fluorescence would decrease) but would not be excluded from bilayers composed of 18:0,22:6-PC (its fluorescence would not decrease). These predictions are born out in Fig. 5. As the cholesterol/phospholipid ratio increases from 0 to 1.0, fluorescence intensity of MC540 decreases for 18:0,18:1-PC but increases slightly for 18:0,22:6-PC bilayers. This implies that MC540 can sense condensing interactions of cholesterol with phospholipids.

We quantified the previous suggestion that MC540 might be used to detect lipid packing [12] by comparing

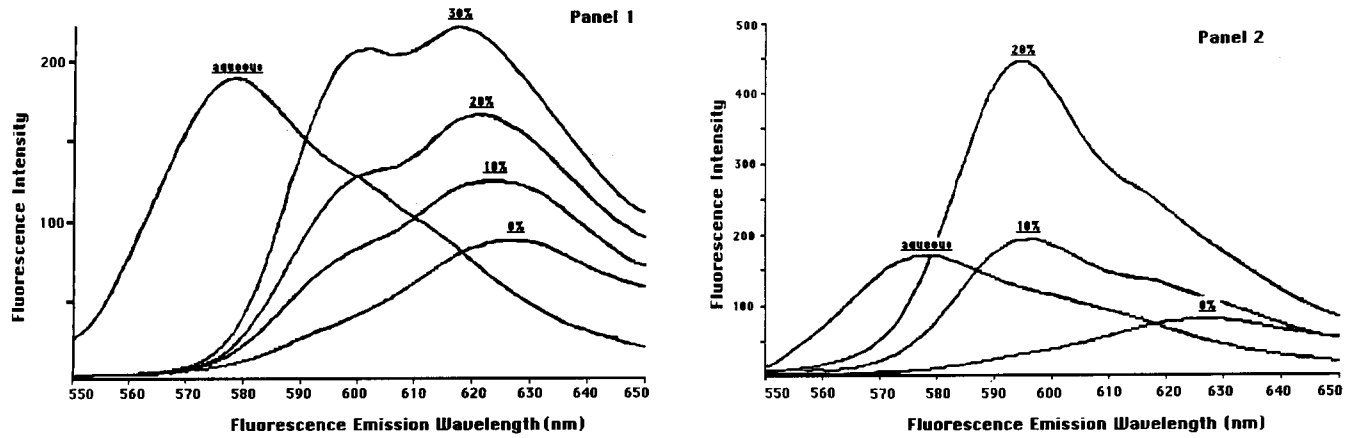

Fig. 2. Representative spectra of MC540 in aqueous solution (control) and in the presence of LUVs composed of 100 mol% DPPC; 10 mol% 18:0,18:1-PC/90 mol% DPPC; 20 mol% 18:0,18:1-PC/80 mol% DPPC; and 30 mol% 18:0,18:1-PC/70 mol% DPPC (Panel 1) and of 100 mol% DPPC; 10 mol% 18:0,22:6-PC/90 mol% DPPC; and 20 mol% 18:0,22:6-PC/80 mol% DPPC (Panel 2). The monotectic lipid mixture demonstrates a liquid-crystalline state emission maxima at about 594 nm and a gel state emission at about 694 nm.

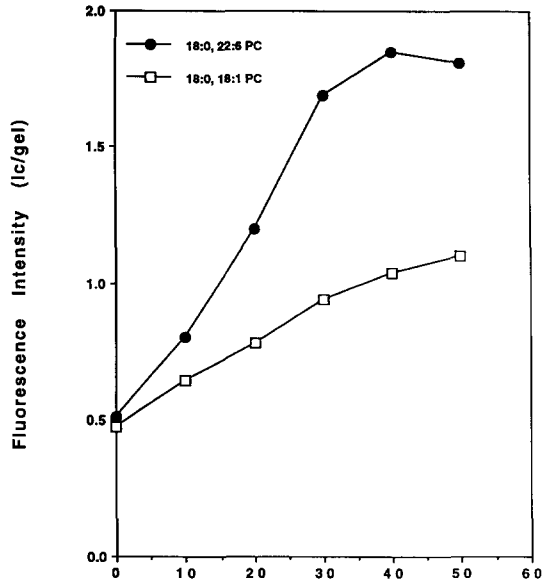

Fig. 3. Ratio of the fluorescence intensity at the liquid-crystalline state emission maxima (594 nm) divided by the fluorescence intensity at the gel state emission maxima (624 nm) as a function of the % of liquid-crystalline state present in mixed chain PC/DPPC bilayers. At the temperature the spectra were obtained (30°C) the percentage of liquid-crystalline state was precisely defined by the mol ratio of the liquid-crystalline (18:0,22:6-PC or 18:0,18:1-PC) to the gel state (DPPC) component.

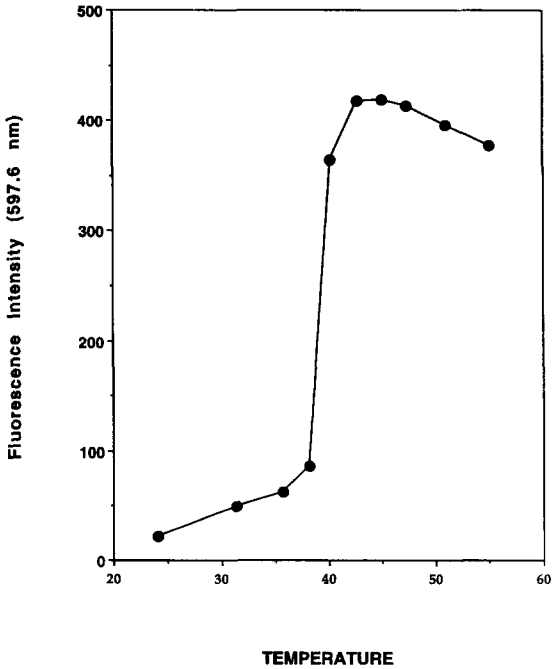

Fig. 4. Fluorescence intensity at the liquid-crystalline state emission maxima of MC540 for DPPC bilayers. Emission spectra were obtained every few degrees as the temperature was slowly decreased through the phase transition of DPPC (41.3°C).

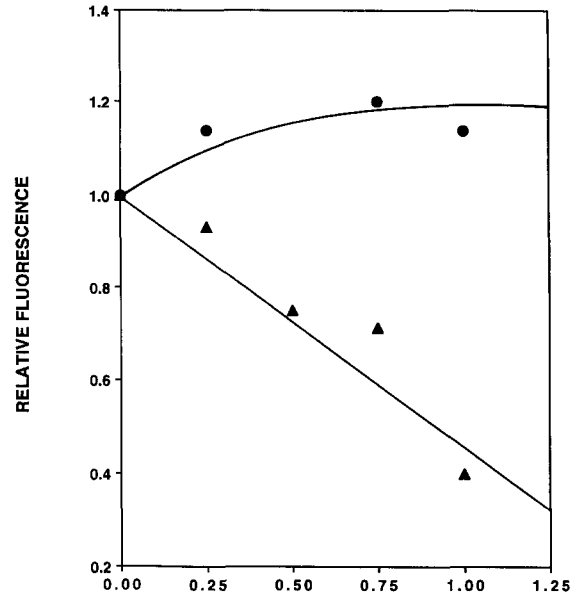

Fig. 5. Relative MC540 fluorescence intensity at the liquid-crystalline state emission maxima (594 nm) with phospholipid-cholesterol vesicles. LUVs were composed either 18:0,18:1-PC (△) or 18:0,22:6-PC (●) and the cholesterol/phospholipid ratio varied from 0 (control) to 1.0.

the fluorescence intensity at the liquid-crystalline peak maxima with the area/molecule determined from pressure/area isotherms of various PCs determined on a Langmuir trough. Fig. 6 reports that there is a good correlation (Pearson's correlation, $P < 0.03$) between the fluorescence and area/molecule data. The area/molecule for 18:0,22:6-PC was determined to be about 115 Å^2 (lateral pressure 32 mN/m).

Since both the EPR-TEMPO and MC540 techniques could accurately assess the relative amounts of liquid-crystalline and gel states in mixed PC bilayers (Figs. 1 and 3), and the MC540 fluorescence intensity could be used to estimate the degree of lipid packing (Fig. 6), we next tested to see if the TEMPO partition coefficient F could also be used to monitor packing in liquid-crystalline PC bilayers. We used several of the same phospholipids reported in Fig. 6. The lipids tested were PCs with either both acyl chains the same (18:1,18:1-PC; 18:2,18:2-PC and α-18:3,α-18:3-PC) or PCs with asymmetric chains (18:0,18:1-PC; 18:0,18:2-PC and 18:0,α-18:3-PC). At the temperature at which the EPR spectra were taken (25°C) all of the lipids are in the liquid-crystalline state. While the MC540 fluorescence showed large differences between the various liquid-crystalline bilayers, there is no significant difference between the measured TEMPO partition coefficients (F) for any of the membranes tested (Fig. 7). Therefore we conclude that MC540 can distin-

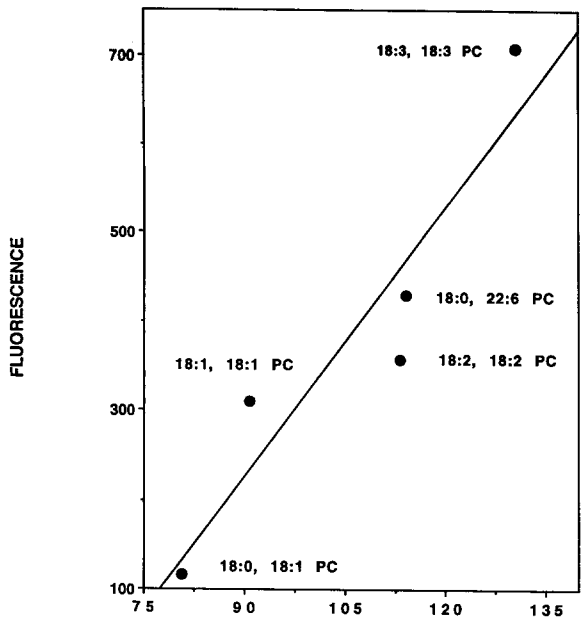

Fig. 6. Area/molecule (Å^2) for various phosphatidylcholines as measured on a Langmuir trough as a function of the fluorescence intensity at the liquid-crystalline state emission maxima (594 nm).

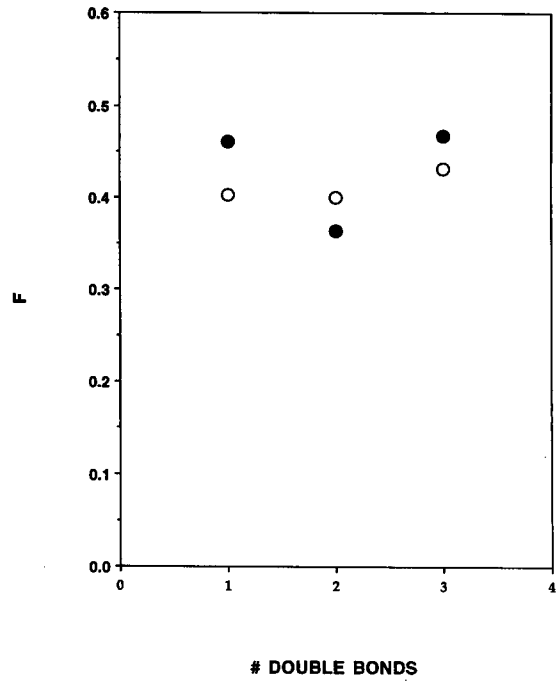

Fig. 7. Partition coefficient F of the spin probe TEMPO as a function of the number of double bonds in symmetric chain PCs (●) (dioleoyl-PC, dilinoleoyl-PC or dilinolenoyl-PC) or mixed chain PCs (○) where the *sn*-1 chain is 18:0 (stearic acid) and the *sn*-2 chain is either 18:1 (oleic acid), 18:2 (linoleic acid) or *sn*-3 (linolenic acid).

guish between liquid-crystalline states composed of different phospholipids and is probably reporting on the packing of lipids in the bilayer while TEMPO can only distinguish liquid-crystalline from gel states and is independent of lipid packing.

Because of its success at distinguishing membrane phases in phospholipid vesicles, we next tested MC540 with a complex biological membrane, the plasma membrane of T27A tumor cells. The tumor cells were modified by DHA incorporation using 18:0,22:6-PC/18:0,22:6-PE (3:1) SUVs. There are two possible mechanisms which may explain DHA uptake, endocytosis and/or fusion. Since the vesicles were incubated for 1 h at 30°C with the T27A cells, endocytosis is a real possibility. We previously demonstrated, however, that DHA-containing phospholipids are highly fusogenic. Also, under identical conditions to the ones used here, we showed that SUVs made from non-fusogenic DPPC were not incorporated into the tumor cells.

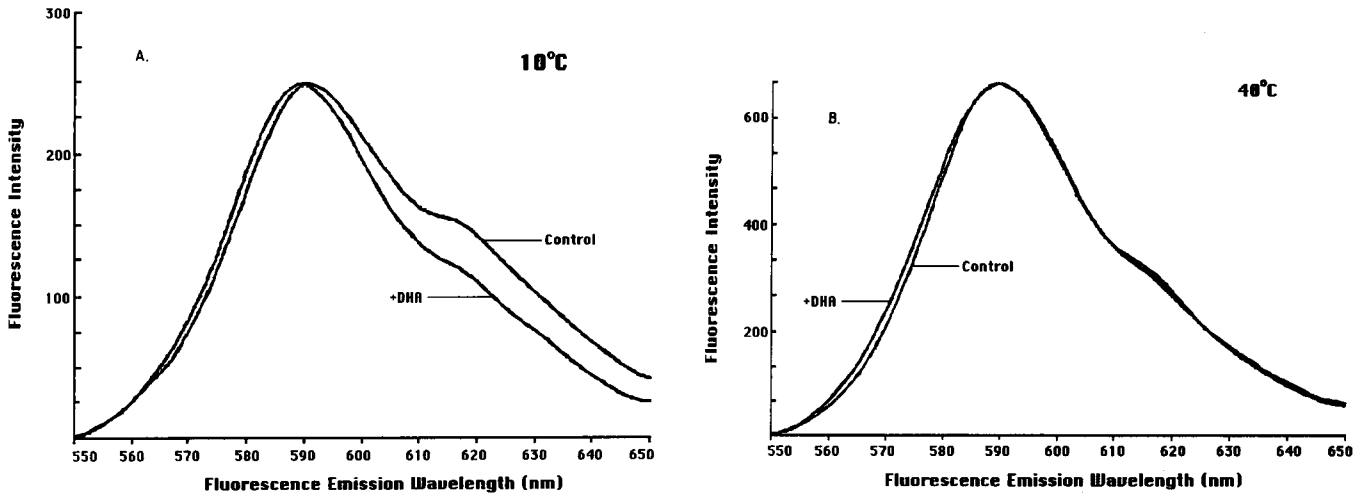

Fig. 8. Fluorescence emission maxima of MC540 in the presence of T27A cells or T27A cells which have been modified by fusion with 18:0,22:6-PC LUVs. The spectra were obtained at 10°C (panel A) or at 40°C (panel B).

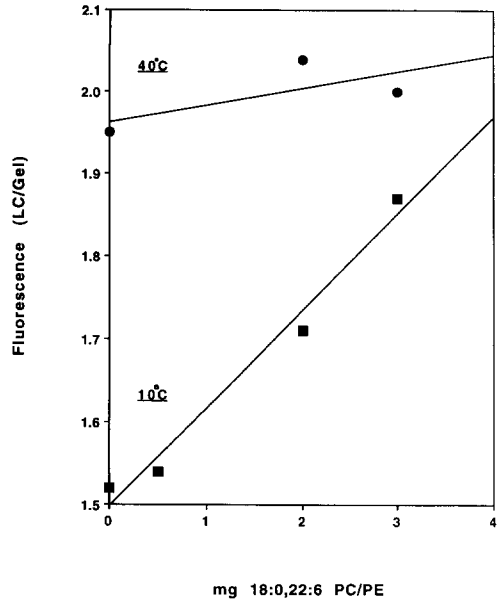

Fig. 9. Ratio of the fluorescence intensity at the liquid-crystalline state emission maxima (594 nm) divided by the fluorescence intentity at the gel state emission maxima (624 nm) as a function of the amount of 18:0,22:6-PE/18:0,22:6-PC incorporated into the T27A tumor cells. Ratios were determined at either 10°C or 40°C.

Regardless of how DHA is incorporated into the tumor cell, it must be located at least in part in the plasma membranes since we have shown that cell permeability [14] and tumor cell epitope distribution [23] are severely affected by DHA. Immediately after incubation the modified tumor cells were separated from the free SUVs. One sample each of both the modified and non-modified cells was then incubated for 30 min with MC540 and the fluorescence emission spectra taken at 10°C, 20°C, 30°C and 40°C. The second cell sample was extracted in $CHCl_3$/MeOH and the DHA content determined by gas chromatography. The non-modified T27A cells did not contain detectable levels of DHA, while DHA represented 13.6% of the modified cells' fatty acids. Spectra for the non-modified (control) T27A cells and the DHA-modified cells at 10°C and 40°C temperature are reported in Fig. 8. The spectra are normalized to the liquid-crystalline peak maxima. The large difference in the gel state region of the spectra between the +DHA and −DHA (control) which is very evident at 10°C disappears as the temperature is increased to 20°C, 30°C and 40°C. This observation is quantified in Fig. 9.

Discussion

MC540 has been frequently used as a probe of membrane structure. Based mainly on indirect observations, the probe has been suggested to "sense the degree of lipid packing in membranes" [12]. Previously it has been demonstrated that membrane fluid state binds MC540 more than does gel state [24]; that sonicated vesicles (SUVs) with looser headgroup packing bind MC540 better than do LUVs [12]; that cholesterol can condense fluid state (DLPC) bilayers 'squeezing' MC540 out of the membrane [12]; and that MC540 can detect 'plasma domains' in a variety of cells [5,12,24]. The implication is that lipid domains of unknown composition are responsible for the uneven distribution of MC540 in some cells. In the experiments presented here we have extended and, using phospholipid vesicles of defined composition, have confirmed and quantified several of the previous general observations on MC540. We have also modified a cell membrane (plasma membrane of T27A cells) by incorporation of DHA, a fatty acid not normally found in the plasma membranes of these cells, and demonstrated that DHA alters membrane liquid-crystal/gel distribution.

Measurement of the ratio of fluorescence intensity at the liquid-crystalline peak maxima (about 594 nm) to the gel state maxima (about 624 nm) for MC540 can give an accurate determination of the percentage of coexisting liquid-crystalline and gel states in two component, monotectic PCs. However, a problem may arise with lipids possessing a large area/molecule (like 18:0,22:6-PC). Membranes made from these lipids exhibit enhanced MC540 fluorescence with intensity at the liquid-crystalline state maximum so intense it is difficult to obtain a meaningful value for the gel state fluorescence intensity. Therefore for membranes made from large area/molecule lipids the ratio of liquid-crystal/gel intensities can only be used for low percentages of liquid-crystalline in gel state membranes. The well established EPR technique of TEMPO partitioning into the liquid-crystalline state of membranes [19], on the other hand, is accurate for any percentage of liquid-crystal/gel states and is independent of the composition of the liquid-crystalline state component. This is illustrated by Fig. 1 where a linear dependence of TEMPO partitioning into 18:0,22:6-PC/DPPC membranes could be measured for 0–100 mol% polyunsaturated phospholipid, whereas the equivalent graph in Fig. 3 based on MC540 fluorescence data is restricted to 0–30 mol% 18:0,22:6-PC.

In contrast to TEMPO partitioning, MC540 is sensitive to liquid crystalline states of different composition. As the area per molecule increases, determined by surface/pressure measurements on a Langmuir trough, the fluorescence at the liquid-crystalline maxima also increases (Fig. 6). From these observations, 18:0,22:6-PC occupies approximately the same area as 18:2,18:2-PC. While the EPR-TEMPO technique can quantitatively distinguish liquid-crystalline from gel states (Fig. 1), it is not sensitive to the different liquid crystalline states (Fig. 7) which can be detected by MC540 fluorescence. EPR, moreover, has the disadvantage of rapid probe reduction by cellular components. MC540 does

not exhibit this problem and so may generate useful numbers with living cells.

MC540 can also be used to monitor the condensing effect of cholesterol on some liquid-crystalline state membranes (Fig. 5). The original observation by Williamson et al. [12] on cholesterol-induced decrease in MC540 fluorescence was based on measurements with liquid-crystalline state, dilauroylphosphatidylcholine (DLPC, 12:0,12:0-PC) bilayers. In the experiments reported here (Fig. 5) we use the more biologically relevant lipids 18:0,18:1-PC and 18:0,22:6-PC. As cholesterol condenses 18:0,18:1-PC bilayers, MC540 is excluded from the membranes and its fluorescence decreases. This is in agreement with the prior DLPC [12] report but is in contrast to our measurements with 18:0,22:6-PC (Fig. 5). Increasing cholesterol does not decrease the fluorescence of 18:0,22:6-PC membranes and we would predict therefore cholesterol would not condense DHA-containing lipids. These fluorescence observations are supported by lipid monolayer studies which have shown that cholesterol condenses oleic acid-containing lipids but has no condensing effect on DHA-containing lipids [22].

Previously we have shown that DHA when added to membranes as either a free fatty acid or when added as a phosphatidylcholine in which DHA has been acylated to the *sn*-2 chain, increases in membrane permeability and fusion were measured [14,15]. The change in these basic membrane properties indicates that DHA must somehow be significantly affecting membrane structure. We could not, however, detect any associated DHA-induced changes in membrane ordering and dynamics as monitored by a variety of fluorescent membrane probes. Furthermore, EPR of 5- and 16-doxyl stearic acids in egg PC membranes failed to detect meaningful changes in order parameter or correlation time following incorporation of 10 mol% 18:0,22:6-PC [25]. It appears that DHA does not significantly modify acyl chain mobility within the interior of membranes already rich in any unsaturated fatty acid. We interpret this to indicate that DHA is probably not increasing membrane permeability and fusion by just increasing general membrane fluidity. Instead we propose that DHA may be altering membrane structure only at localized regions, perhaps introducing defects between DHA-rich and DHA-poor domains. The possible interaction of fatty acyl chains with cholesterol, a major component of animal plasma membranes, suggests that the addition of DHA to these membranes may produce an oleic acid-rich, cholesterol-rich domain and a DHA-rich, cholesterol-poor domain. Various membrane proteins could then be distributed between these domains which may be detectable by MC540 fluorescence.

The experiment with the T27A tumor cells implies that MC540 may be used to distinguish subtle differences between the liquid-crystalline/gel state ratios for cells of nearly identical composition. At 40°C where the vast majority of phospholipids are in the liquid-crystalline state, there is no observable difference between the response to MC540 with DHA-modified and unmodified T27A cells. In contrast, at 10°C there is clear separation between the liquid-crystalline and gel states for the T27A control cells which is substantially diminished by DHA. Since the two cell populations are identical except for the incorporated DHA, the difference noted in the degree of coexisting liquid-crystalline and gel states must be due to the presence of DHA. DHA has modified the tumor's plasma membrane perhaps by the formation of unique domains that can influence membrane function under physiological conditions.

We are currently pursuing the possibility that DHA induces lateral phase separation in membranes by measuring the distribution of plasma membrane epitopes for tumor cells enriched to different extents with DHA. We are pursuing the possibility that lateral phase separation induced by DHA may expose different epitopes on the tumor cell membrane surface making the tumor cells more susceptible to immunosurveillance by the host cells.

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References

1 Waggoner, A.S. (1979) Annu. Rev. Biophys. Bioeng. 8, 47–68.
2 Kinally, K.W., Tedeschi, H. and Maloff, B.L. (1978) Biochemistry 17, 3419–3428.
3 Salama, G. and Morad, M. (1976) Science 191, 485–487.
4 Chance, B. and Baltscheffsky, M. (1975) in Biomembranes (Eisenberg, H., Katchalski-Katzir, E. and Manson, L.A, eds.), Vol. 7, p. 33, Plenum Press, New York.
5 Schlegel, R.A., Phelps, B.M., Waggoner, A., Terada, L. and Williamson, P. (1980) Cell 20, 321–328.
6 Phelps, B.M., Williamson, P. and Schlegel, R.A. (1982) J. Cell Physiol. 110, 245–248.
7 Schlegel, R.A., Phelps, B.M., Cofer, G.P. and Williamson, P. (1982) Exp. Cell Res. 139, 321–328.
8 Valinsky, J.E., Easton, T.G. and Reich, E. (1978) Cell 13, 487–499.
9 Ojcius, D.M., Jiang, S., Persechini, P.M., Storch, J. and Young, J.D.-E. (1990) Mol. Immunol. 27, 839–845.
10 Lelkes, P.I. and Miller, I.R. (1980) J. Membr. Biol. 52, 1–15.
11 Pohl, G.W. (1976) Z. Naturforsch. 31c, 575.
12 Williamson P., Mattocks, K. and Schlegel, R.A. (1983) Biochim. Biophys. Acta 732, 387–393.
13 Dratz, E.A. and Deese, A.J. (1985) in Health Effects of Polyunsaturated Fatty Acids in Seafoods (Simopoulos, A.P., Kifer, R.R. and Martin, R.E., eds.), pp. 319–351, Academic Press, Orlando, FL.
14 Stillwell, W., Ehringer, W. and Jenski, L.J. (1993) Lipids, in press.
15 Ehringer, W., Belcher, D., Wassall, S.R. and Stillwell, W. (1990) Chem. Phys. Lipids 54, 79–88.

16 Wassall, S.R., McCabe, M.A., Griffith, G.L., Ehringer, W.D. and Stillwell, W. (1992) Biophys. J. 61, A240.
17 Keough, K.M.W., Griffin, B. and Kariel, N. (1987) Biochim. Biophys. Acta 902, 1–10.
18 Comfurius, P. and Zwaal, R.F.A. (1977) Biochim. Biophys. Acta 488, 36–42.
19 Shimshick, E.J. and McConnell, H.M. (1973) Biochemistry 12, 2351–2360.
20 Coolbear, K.P., Berde, C.B. and Keough, K.M.W. (1983) Biochemistry 22, 1466–1473.
21 Deese, A.J., Dratz, E.A., Dahlquist, F.W. and Paddy, M.R. (1981) Biochemistry 20, 6420–6427.
22 Demel, R.A., Van Kessel, G.W.S.M. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 266, 26–40.
23 Pascale, A.A., Ehringer, W.D., Stillwell, W., Sturtevant, L.K. and Jenski, L.J. (1993) Nutr. Cancer 19, in press.
24 Williamson, P.L., Massey, W.A., Phelps, B.M. and Schlegel, R.A. (1981) Mol. Cell. Biol. 1, 128–135.
25 Wassall, S.R., McCabe, R.Y.C., Ehringer, W. and Stillwell, W. (1992) J. Biol. Chem. 267, 8168–8174.