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Merocyanine interaction with phosphatidylcholine bilayers

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Merocyanine (MC 540) is a fluorescent probe whose optical properties depend on the environmental polarity. In the presence of lipid bilayers, MC 540 binds to the membrane surface while simultaneously changing its fluorescence properties. Previous studies have shown that the fluorescence of merocyanine depends upon the lipid packing in the membrane. We measured the partitioning of MC 540 and its fluorescence properties in the presence of phosphatidylcholine membranes. We found that the fluorescence of MC 540 shows, as expected, a major change around the main phase transition of phosphatidylcholine membranes. However, instead of a step-like increase of fluorescence, the maximum at phase transition was observed. We were able to explain our data by combining two effects; dependence of MC 540 fluorescence on temperature and lipid fluidity. In addition, we established that the increase of the fluorescence intensity in the presence of lipid bilayers in the fluid state is due to the elevated partitioning of the probe into the lipid phase. The partition of MC 540 into the fluid membrane does not depends on the dye concentration in the aqueous phase. When lipid was in the gel phase the partitioning of the dye increased with its bulk concentration, whereas the fluorescence intensity remained unchanged. We conclude, therefore, that MC 540 forms nonfluorescent complexes when in the gel lipid membrane.

Introduction

MC 540 (5-[(sulfonyl-2(3H)-benzoxazoylidine)-2-butenyldene]-1,2-dibutyl-2-thiobarbarbituric acid) is a chromophore with a negative charge. Its optical properties depend strongly on the environment. The dye is particularly sensitive to changes in polarity. When dissolved in water its absorbance and fluorescence emission is low compared to that in hydrocarbon solvents [1]. Those properties, combined with the ability of the probe to interact with lipid surfaces make this dye very attractive in a variety of membranes studies.

MC 540 was first used to measure cellular transmembrane potential [2–4]. Later, it was applied to differentiate between cell types (for a review, see Sieber et al. [5]) as well as for selective killing of malignant cells [5–7]. Later studies on model membranes correlated fluorescence efficiency of MC 540 with the membrane organization. Lelkes et al. [8,9] studied the dye location within the lipid bilayer and its sensitivity to membrane structure. Using calorimetry and spectroscopic measurements, they showed that the dye is located in the interfacial region of membranes. They also suggested that the dye forms domains in lipid bilayers when introduced in high concentrations. This particular result agrees with earlier findings of Waggoner and Grinveld [3]. Later experiments carried by Williamson et al. [10] indicated that the fluorescence intensity of the dye is sensitive to the lipid packing in bilayers. Their experiments on lipid vesicles showed that the fluorescence of MC 540 is enhanced in the presence of the disordered or fluid membranes.

There are many factors, such as partitioning, temperature, polarity of the environment, etc., that effect the fluorescence characteristics of MC 540. These factors complicate the use of MC 540 as a fluidity probe. While the studies listed above were concerned with different aspects of MC 540 interaction with lipid bilayer, a more comprehensive understanding of merocyanine fluorescence in the presence of lipid membranes is needed. In this paper we present our results which would contribute to a better understanding of MC 540 fluorescence properties in the presence of phosphatidylcholine bilayers under different physical conditions.

Materials and Methods

Reagents. Merocyanine (MC 540) was purchased from Molecular Probes (Eugene, OR). Dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC), dioleoyl-L-α-phosphatidylcholine (DOPC), dilauroyl-L-α-phosphatidylcholine

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(DLPC) and distearoyl-L-α-phosphatidylcholine (DSPC) were obtained from Avanti Polar Lipids (Pelham, AL). All other chemicals were from Fisher Scientific (Fairlawn, NJ). All experiments were performed in 0.14 M phosphate buffer (pH 7.4).

Vesicle preparation. Lipid dissolved in chloroform was dried under nitrogen and dispersed with phosphate buffer at temperatures above its main phase transitions. The multilamellar vesicle suspensions were used as stock solutions. Unilamellar vesicles were formed by extrusion technique [11,12].

Partitioning measurements. The partition of MC 540 between the membrane and aqueous phase was determined by the estimation of MC 540 concentration in buffer after incubation with multilamellar lipid vesicles. Samples containing MC 540 and 1 mg/ml multilamellar vesicles, in 150 mM NaCl buffered with 5 mM Tris-MA to pH 7.4, were incubated for 10 min and were centrifuged to separate dissolved dye in the supernatant from the lipid. The fluorescence intensities of 50 μl, 100 μl and 150 μl of supernatant dissolved in ethanol were measured. Each experiment was repeated at least three times. Results were compared to that of control samples (without vesicles) after the same treatment. The amount of lipid in the supernatant after centrifugation was estimated with a standard phosphate test [13]. The measured amount of phosphate in supernatant did not significantly exceed the background level (i.e., sample without vesicles).

Fluorescence measurements. Fluorescence intensities of MC 540 were recorded on an SLM 8000 fluorometer at excitation and emission wavelengths 540 nm and 585 nm, respectively. The temperature of the sample was maintained by a circulating water bath. The lipid sample was incubated for 15 min prior to the addition of the dye. Vesicles and MC 540 were incubated for an additional 10 min and then the fluorescence intensity was monitored for at least 1 min. In addition, at each temperature the fluorescence of MC 540 was measured for at least five probe concentrations, in order to check the linearity of the dependence of fluorescence on merocyanine concentration. The result was averaged by calculating the slope of fluorescence versus dye concentration. The slope was calculated using the least-square method.

In all fluorescence experiments the lipid concentration in the sample was 0.05 mg/ml. We did not apply inner filter and light scattering corrections, since the estimated error was no higher than 10% of the measured fluorescence intensities.

Results and Discussion

Williamson et al. [10] found that fluorescence of MC 540 depends upon lipid membrane fluidity and packing. They observed that when MC 540 suspension was titrated with DLPC and DPPC vesicles, the fluorescence intensity was enhanced in the presence of fluid phase DLPC lipid vesicles. Our experiments, using a number of phosphatidylcholines that are either in the fluid phase or in the gel phase at room temperature at a fixed probe concentration of $3 \cdot 10^{-5}$ mg/ml, gave results similar to those presented by Williamson et al. [10]. Merocyanine in the presence of lipid membranes in the fluid phase yields a higher fluorescence intensity (data not shown). The emission spectra of MC 540 are also found to be different in the presence of fluid or gel-state bilayers, in agreement with that reported by Williamson et al. [10]. The emission maximum was enhanced and shifted in the direction of the longer wavelength, when MC 540 was added to fluid-phase lipid vesicles.

Lelkes et al. [8], studied the absorbance of MC 540 exposed to a variety of lipid vesicles at different temperatures. In their experiments absorbance increased rapidly at a temperature when the lipid main phase transition occurs.

Taking into account results obtained by Williamson et al. [10] and Lelkes et al. [8,9] we expected that the fluorescence emission spectrum would have behaved similar to that of the absorbance in Lelkes experiment [8], i.e., when incubated in the presence of lipid vesicles at temperatures below main phase transition, the MC 540 fluorescence should be much lower than that at temperatures above main phase transition. The increase of the fluorescence should have a step-like pattern with a rapid increasement of intensity at the main lipid phase-transition temperature.

Temperature effect

We measured the fluorescence intensity of MC 540 at constant lipid and dye concentrations but at various temperatures. Lipid vesicles were incubated at desired temperatures for 15 min to equilibrate the sample. Then MC 540 was added from an ethanol stock solution and the sample was again incubated in the dark to ensure a complete partition of the probe. The progress of the partition and the time required to complete the process was monitored in separate experiments by recording the fluorescence emission maximum at 585 nm. We found that the fluorescence intensity stabilized within 10 min after addition of the probe. The ethanol concentration was always lower than 0.5% by volume.

The fluorescence intensity of MC 540 added to DMPC, DPPC and DSPC vesicle suspensions at varying temperatures is shown in Fig. 1. Data shown in Fig. 1 follows the expected pattern with respect to the sharp increase of fluorescence intensity at the gel–liquid phase transition. However, we observed a number of unexpected features. The fluorescence intensities measured for DMPC were much higher than that for DSPC and DPPC when measured at the fluid

phase. Furthermore, the fluorescence data show a maximum at the temperature of the main lipid phase transitions instead of the expected step-like increase.

We suspected that the fluorescence intensity of MC 540, when in the lipid bilayer, depended strongly on the temperature. The same effect has been observed for a number of other fluorescence dyes [14]. Consequently, we measured the fluorescence intensities of MC 540 in the presence of vesicles formed by lipids which are in the fluid phase throughout the entire range of temperatures measured. The data obtained for DOPC are shown in Fig. 2. A similar result was obtained for DLPC (data not shown). In both cases the fluorescence intensity of MC 540 decreased with increased temperature.

This temperature-dependence might be caused by changes in the quantum efficiency of the probe [14] or by an alteration of the partitioning between bulk solution and the lipid bilayer. In order to establish the temperature-dependence of MC 540 quantum efficiency, we measured the MC 540 fluorescence at various temperatures in the ethanol solution. The polarity of ethanol is similar to that which merocyanine experiences in the lipid membrane [9]. As shown in Fig. 2, the fluorescence of MC 540 decreased linearly with temperature. The linear relationship between fluorescence intensity and temperature enables us to apply a correction for this effect when the probe is in the presence of lipid membrane. The dependence of MC 540 fluorescence on temperature provides a satisfactory explanation for effects described above. The high fluorescence intensity of the probe in the presence of

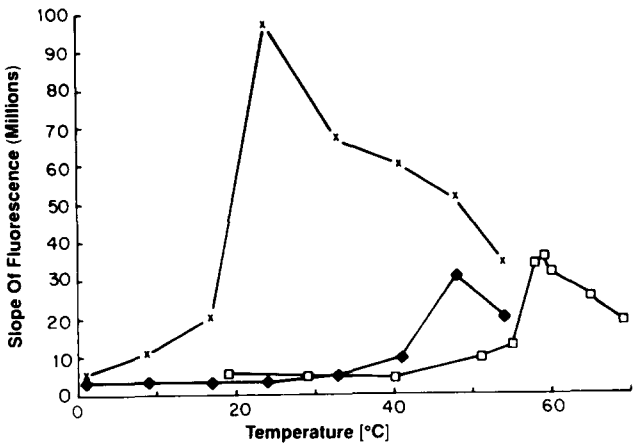

Fig. 1. MC 540 fluorescence at 585 nm as a function of temperature. The fluorescence is expressed in terms of slope calculated from the plot of fluorescence intensity as a function of probe concentration in the aqueous phase. The fluorescence in the presence of DMPC vesicles (×), DPPC (♦) and DSPC (□) are shown. All vesicles were unilamellar and suspended in the phosphate buffer (pH 7.4). The MC 540 and lipid concentrations were $3.33 \cdot 10^{-4}$ mg/ml and 0.05 mg/ml, respectively. Excitation wavelength was 540 nm.

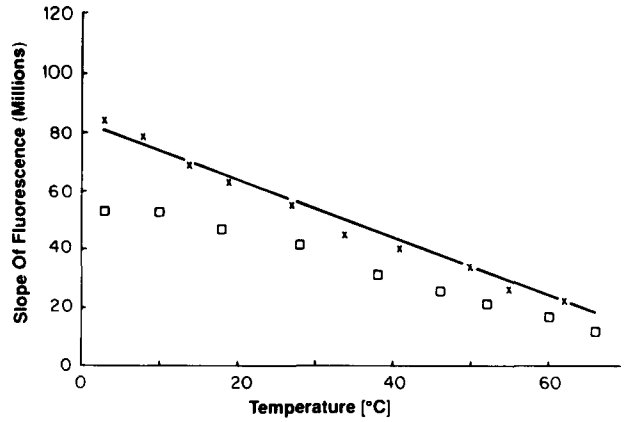

Fig. 2. Fluorescence of MC 540 in the presence of DOPC unilamellar vesicles (□) and ethanol solution (×) as a function of temperature. The solid line represents a least-squares data approximation.

DMPC vesicles at the main phase transition, as compared to DPPC and DSPC membranes, is now understanable. The DMPC main phase transition is at a low temperature (24°C) when MC 540 fluorescence is high. DPPC (42°C) and DSPC (55°C) phase transitions are at higher temperatures, therefore the MC 540 fluorescence intensity is low, exclusively due to a temperature effect.

The fluorescence intensity maximum at the main phase transition of lipid (Fig. 1) is also understandable. The superposition of a temperature effect on the probe's quantum efficiency and change of membrane fluidity at the main phase transition produce the apparent fluorescence maximum.

When fluorescence intensities are corrected for the temperature effect, our results are consistent with predictions based on previous data [8–10]. We corrected the MC 540 fluorescence intensity at a given temperature by dividing it by the fluorescence of the probe in ethanol measured at the same temperature. As expected, the corrected MC 540 fluorescence intensity in the presence of DOPC vesicles as a function of temperature does not change as shown in Fig. 3. Similar results were obtained for DLPC (data not shown).

Fig. 4A and 4B show the corrected fluorescence obtained for MC 540 in the presence of DMPC and DSPC vesicles. The fluorescence maximum at the main phase transition (Fig. 1) practically disappeared after correction for the temperature effect. The corrected fluorescence intensities at gel and liquid phases are significantly different, a result similar to that obtained by Williamson et al. [10]. Furthermore, there is no significant difference between the MC 540 fluorescence in the presence of a fluid membrane formed from DMPC, DSPC or DPPC (data not shown). Therefore, the fluidity of lipid bilayer is indeed a decisive factor determining MC 540 fluorescence in the presence of phosphatidylcholine membranes.

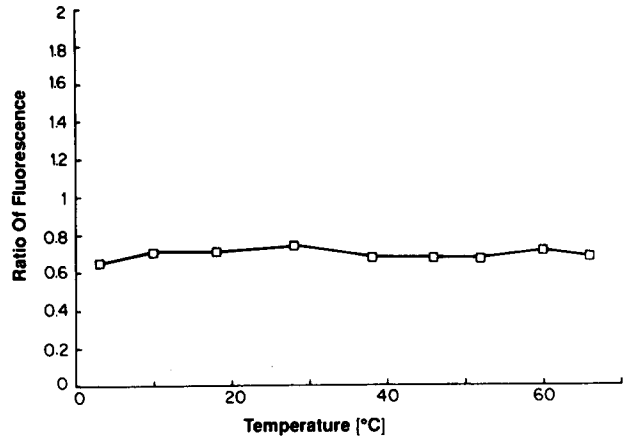

Fig. 3. MC 540 fluorescence corrected for the temperature effect in the presence of DOPC unilamellar vesicles. The fluorescence intensity of MC 540 in the presence of DOPC was divided by the fluorescence obtained when the probe was in ethanol (Fig. 2).

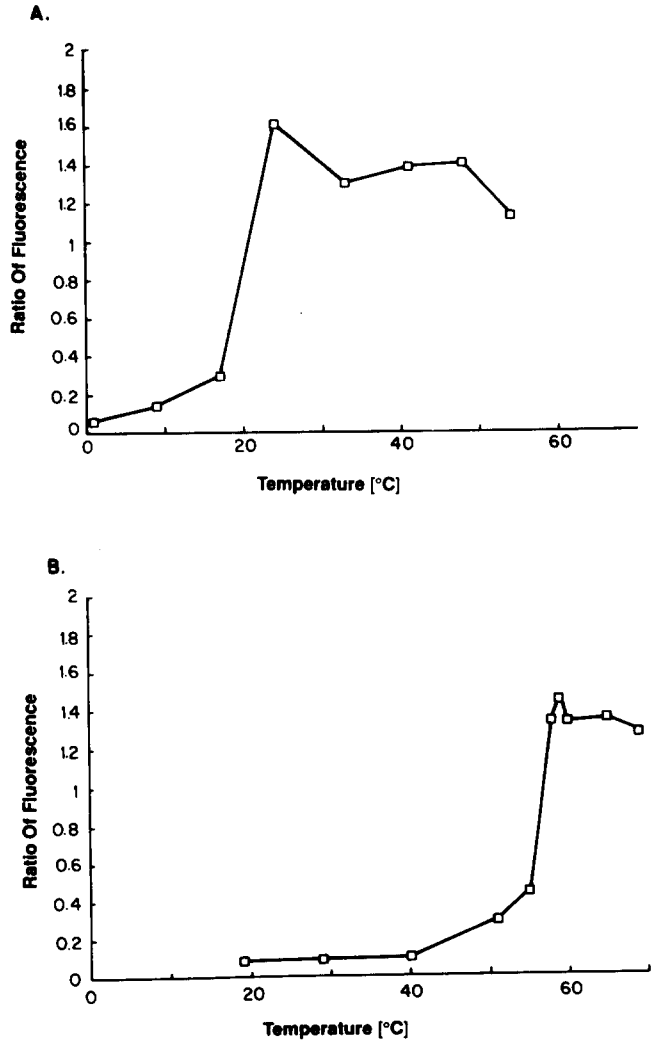

Fig. 4. Sections A and B represent the fluorescence of MC 540 corrected for temperature effect in the presence of DMPC and DSPC unilamellar vesicles, respectively.

The partition effect

The results presented above have confirmed previous observations [8–10] that the fluorescence intensity of MC 540 is enhanced when the probe is incubated with membranes in the fluid phase. There are two possible explanations for this observation: the quantum yield of MC 540 incorporated into the lipid bilayer might increase when membrane is in the fluid phase, or the probe partitioning between water and lipid is higher when the lipid is in the fluid phase. The apparent quantum efficiency might change as a result of the probe's relocation during lipid phase transition.

To establish which of these two factors results in MC 540 fluorescence enhancement, we studied partitioning of the probe between water and lipids when in different phases. To determine the partition, we measured dye concentration in the aqueous phase after incubation with a multilamellar lipid vesicle suspension (1 mg/ml). The amount of fluorophore residue with lipid membranes was then estimated as described in Materials and Methods. We used DOPC and DPPC vesicles in the partition experiments because they formed membranes in liquid and gel phase, respectively, when incubated at room temperature. Results of the partition experiments are shown in Fig. 5. When MC 540 is incubated with DOPC vesicles (fluid phase), the amount of probe associated with lipid bilayer is proportional to its concentration in the aqueous phase. Approx. 60% of dye is associated with lipid membrane and this percentage does not vary within the used range of probe concentrations. MC 540 in the presence of DPPC vesicles (gel phase) behaves differently. When the concentration of MC 540 in water is low, less than $2 \cdot 10^{-4}$ mg/ml, no detectable association with lipid was observed. When the total dye concentration in the sample increases above this level, the amount of MC

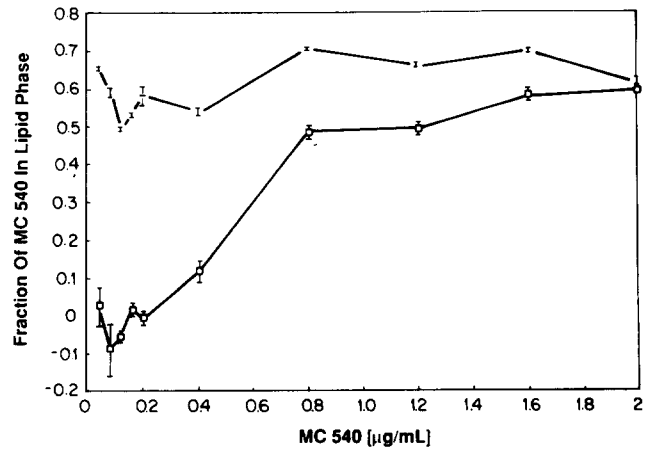

Fig. 5. The partitioning of MC 540 into the lipid matrix. The partition is expressed as ratio of probe incorporated into lipid bilayer to the total probe concentration. Squares and bars represent amount of MC 540 binding to DPPC and DOPC multilamellar vesicles, respectively. All data were collected at room temperature.

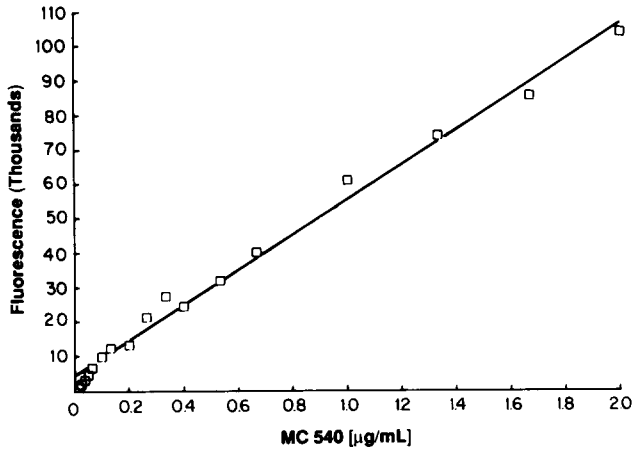

Fig. 6. The MC 540 fluorescence intensity as a function of dye concentration in phosphate buffer at room temperature.

540 associated with DPPC vesicles increases to that of DOPC at higher dye concentrations (Fig. 6).

Since MC 540 fluorescence does not increase with total dye concentration in the presence of lipid vesicles in the gel state, we are assuming that MC 540 forms nonfluorescent complexes (dimers, oligomers or domains) in lipid membrane. The existence of such domains has been proposed previously [4,9,15,16].

In summary, MC 540 fluorescence intensity in the presence of lipid vesicles depends on a number of factors other than lipid state such as temperature and total probe concentration. When all of these factors are taken into account the relation between the state of lipid and MC 540 fluorescence intensity is simple. The fluorescence intensity of MC 540 in the presence of a fluid lipid membrane is significantly enhanced. At low probe concentration and after correction for temperature effect, the difference in the fluorescence intensities is due only to the difference in the partition characteristics of the dye into lipid bilayers. However, in high probe concentrations, the picture is more complex.

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